Intraorgan monitoring for detection of ischemia and rejection

Lars Wælgaard

The Acute Clinic, Department of Anesthesiology and Intensive Care Medicine and

The Intervention Center,

Oslo University Hospital – Rikshospitalet

Institute for Clinical Medicine,

Medical Faculty,

University of Oslo

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Content:

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Abbrevations

ALT	Alanine aminotransferase
ANOVA	Analysis of variance
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
CRRT	Continuous renal replacement therapy
CI	Cardiac index
CVP	Central venous pressure
DAMP	Danger-associated molecular pattern
DC	Dendritic cell
DO_2	Oxygen delivery
ERC	Endoscopic retrograde cholangiography
ICU	Intensive care unit
IL	Interleukin
IR	Ischemia and reperfusion
IRI	Ischemia and reperfusion injury
kDa	kiloDalton
H^+	Hydrogen ion or proton
HAT	Hepatic artery thrombosis
LSEC	Liver sinusoidal endothelial cell
MAP	Mean arterial pressure

MWCO	Molecular weight cut- off
NAD^+	Nicotinamide adenine dinucleotide (oxidized form)
NADH	Nicotinamide adenine dinucleotide (reduced form)
NF-κB	Nuclear factor kappa-B
NO	Nitric oxide
OLT	Orthotopic liver transplantation
PAMP	Pathogen-associated molecular pattern
PCO_2	Partial pressure of carbon dioxide
PCR	Polymerase chain reaction
PRR	Pattern recognition receptor
PSC	Primary sclerosing cholangitis
PDH	Pyruvate dehydrogenase
PMN	Polymorph nuclear neutrophiles
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
TEG	Thromboelastography
TLR4	Toll-like receptor 4
TNF-α	Tumour necrosis factor- alpha
UW solution	University of Wisconsin solution
VO_2	Oxygen consumption

Definitions

Cut off	The pore size of the microdialysis membrane described as the molecular weight in Daltons that can pass the semi-permeable membrane
Ischemia	Blood supply insufficient to maintain aerobic metabolism
IRI	The inflammation and thereby damage occurring when a tissue is reperfused after a period of ischemia
Microdialysate	The fluid that is recovered over the semipermeabel microdialysis membrane from the interstitial fluid
Microvial	Plastic vial for collection of microdialysate
Recovery	The ratio between the concentration of a substance in the microdialysate and the interstitial concentration
Reperfusion	Return of blood supply to a tissue that has sustained an ischemic period

Erratum

p. 15 line 10: «and» should be deleted

p. 15 line 11: increase**s**

p. 18 line 12: «with» should be deleted

p. 19 last line: «with» should be inserted after «treatment»

p. 20 line 12 and 13: the sentence ”HAT has a mortality rate of approximately 30%, whereas for portal vein thrombosis the mortality is 70%” is written twice.

p. 24 line 18: «of» should be inserted before «a multitude of…»

p. 31 line 7: enter**s**

p. 33 line between line 19 and 20: a ”4” should be deleted

p.51 line 11 ”membranes” should be inserted after ”cut off”.

Reference 4: First author is Daniel De Backer, not "De BD".

List of papers

The thesis is based on three papers:

Study I

Tissue gas tensions and tissue metabolites for detection of organ hypoperfusion and ischemia

Lars Wælgaard, Berit Marie Dahl, Gunnvald Kvarstein and Tor Inge Tønnessen

Accepted for publication in Acta Anaesthesiologica Scandinavica

Study II

Microdialysis for monitoring inflammation: efficient recovery of cytokines and anaphylatoxins provided optimal catheter pore size and fluid velocity conditions

Lars Wælgaard, Anne Pharo, Tor Inge Tønnessen and Tom Eirik Mollnes

Scand J Immunol. 2006 Sep;64(3):345-52.

Study III

Microdialysis monitoring of liver grafts by metabolic parameters, cytokine production and complement activation

Lars Wælgaard, Ebbe Billmann Thorgersen, Pål-Dag Line, Aksel Foss, Tom Eirik Mollnes and Tor Inge Tønnessen

Transplantation. 2008 Oct 27;86(8):1096-103.

Introduction

After major surgery like transplantations, robust tools to monitor and detect occult deterioration in organ function are crucial. Compromised perfusion or inflammation may threaten organ function and thereby the whole organism. Patients may for different reasons be in danger of ischemia (trauma, occult bleeding, thrombosis, insufficient vascular anastomosis, hypovolemia, hypoxia and infections). The transplanted patient is in addition threatened by immunologically incidents and severe graft rejection. All transplant procedures include ischemia-reperfusion injury (IRI) and may trigger activation of an inflammatory posttransplant reaction that may vary from hardly measurable to organ primary non-function.

Clinical monitoring is today based on systemic parameters like arterial and central venous blood pressure (CVP), pulse frequency, systemic oxygen saturation and preload/fluid responsiveness (1;2). These parameters, however, do not reflect regional and microvascular organ blood supply or cellular function. The redistribution of blood flow during hemorrhage gives priority to brain and heart which may lead to “silent ischemia” in visceral organs (3-7). The liver, intestine and the kidney may be severely hypoperfused, but this may not be reflected by whole body hemodynamics or systemic blood samples.

Orthotopic liver transplantation is now an established procedure in the treatment of patients with irreversible liver diseases with no other possible treatment option (8). Due to better surgical techniques, recent developments in anesthesiology and intensive care treatment and more effective immune suppression/anti-rejection treatment, the one year survival in Europe has reached 82% (graft survival 75%) and five-year survival 71% (graft survival 63%) (9). The respective patient survival numbers for Norway are 90% and 83% (10). In 1980 the one-year survival was 28 % worldwide (11)!

Despite this progress, acute rejection is encountered in about 30% - 60% of the patients (12;13), and too many grafts are still lost because of vascular complications like hepatic artery thrombosis, biliary complications, infections, primary non-function of the transplant, recurrence of the primary liver disease and chronic rejection (14) leading to retransplantation or death. There is an obvious need for more specific diagnostic tools to detect these major complications to save as many grafts as possible.

Background

Energy formation from aerobic and anaerobic carbohydrate metabolism

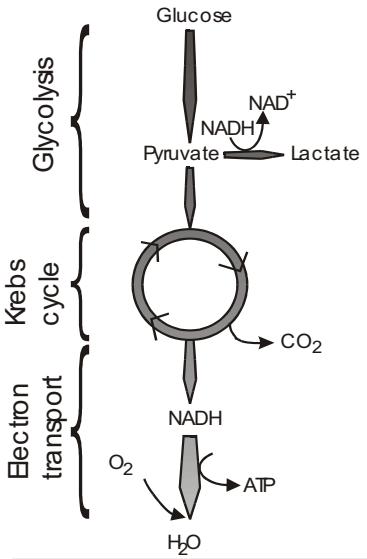


Figure1: Intermediary metabolism in glycolysis, Krebs cycle and electron transport chain

The energy released by the breakdown of glucose is chemically transformed into molecules of adenosine-tri-phosphate (ATP) by a series of enzyme-dependent processes through glycolysis, the citric acid cycle (Krebs cycle) and the electron transport chain (see figure 1). All cells can use glucose as its energy substrate, and some cells (for instance liver, myocardial and skeletal muscle cells) can also utilize lactate, ketones and fatty acids.

The glycolytic pathway from glucose to the intermediate pyruvate provides a rapid but small amount of energy, i.e. 2 ATP molecules per molecule of glucose. If oxygen is present, pyruvate is further decarboxylated through different steps of the citric acid cycle to the end products water and carbon dioxide. For each round in the Krebs cycle two CO_2 molecules are released, while one proton and two electrones are

transferred to NAD^+. This transfer of electrons into the electron chain is a very efficient process that provides 36 molecules of ATP per glucose molecule. The last step of the metabolic chain, where NADH is oxidized to NAD^+, takes place when oxygen is present. To prevent NADH from accumulation in lack of oxygen, pyruvic acid is reduced to lactic acid. The toxic co-factor NADH is hereby converted to NAD^+ which enables the glycolysis to continue when the Krebs cycle and electron chain transport is halted. So, the amount of ATP produced under ischemic conditions is small enabling the cells to survive only for a short amount of time. In brain cells this give energy for approximately five minutes of no flow. In skeletal muscle there are energy reserves like creatine phosphate that can produce ATP and keep the muscle alive for 4 – 12 hours. Heart cells die within 30 - 45 minutes of lack of oxygen, the liver, kidney and intestine sustain approximately 30 – 60 minutes (15).

Metabolism during ischemia

The accumulation of lactic acid under anaerobic conditions rapidly leads to an intracellular acidosis. Since anaerobic metabolism does not provide sufficient amount of ATP, the cells will rapidly consume their ATP which further contributes to the acidification.

The cells would be destroyed if the acidification was not limited by effective intracellular buffer systems. The most important buffers are proteins and bicarbonate and to a lesser extent inorganic phosphate. It is mainly the amount of histidine that determines the buffer capacity of proteins. Histidine is the only amino acid with pKa near physiological pH. Because of the higher concentration of proteins intracellularly compared to plasma and the interstitium, the buffering of protons by proteins inside the cell is greater than outside (16).

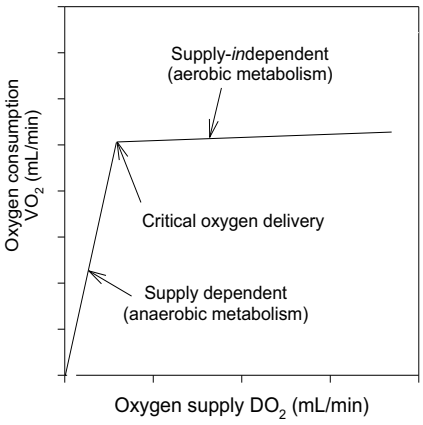


Figure 2: The relation between oxygen consumption and oxygen delivery in an organ follows a dual line. Due to increased oxygen extraction moderate decreases in oxygen delivery will not change the oxygen consumption. When blood flow becomes seriously reduced, additional reductions in oxygen delivery lead to a rapid drop in oxygen consumption (Figure used with permission from Acta Anaest Scand).

The protons liberated during anaerobic metabolism also react with HCO_3^- and form CO_2 and H_2O. Except for nerve cells with a high electric resting potential, skeletal muscle and myocardial cells with a lower intracellular HCO_3^-, the concentration of HCO_3^- is about the same intracellularly and extracellularly. The ability of CO_2 to pass cell membranes and be and carried away and be expired by the lungs increase the buffer capacity of HCO_3^- substantially. During ischemia, however, when blood supply is limited, CO_2 will accumulate in the tissues. This is the basis for using CO_2 to diagnose intracellular acidosis and ischemia (17). CO_2 diffuses through the cell membrane according to the transmembrane gradient so measurements of PCO_2 in the interstitium, on the surface of solid organs, or inside the intestinal lumen correlate well with the intracellular PCO_2.

Even a moderate reduction in blood flow and thereby reduced DO_2 are compensated by increased O_2 extraction to maintain normal cellular functions. The oxygen consumption (VO_2) under aerobic conditions is termed ”supply independent”. Below a “critical” level, defining the anaerobic threshold, however, the tissues are no longer able to compensate by increased

extraction, the consumption (VO_2) has become "supply dependent" and decreases linearly with the reduction in DO_2 as demonstrated in figure 2 (18).

Increased oxygen extraction allows each volume unit of venous blood to receive more CO_2 in return. Thus, even a moderate reduction in the organs blood supply will increase venous PCO_2. Since there is a PCO_2 gradient from the tissue to venous blood, an increase in venous PCO_2 occurs concomitantly with an increase in tissue PCO_2.

Parameters for intraorgan monitoring of ischemia

Systemic calculations of VO_2 and DO_2 are inadequate in detecting occult tissue hypoxia in critically ill patients. Tissue gazes like PCO_2 and PO_2 reflect ischemia. It can be measured directly in expired air, in arterial and venous blood, intraluminally in gastrointestinal tractus, on the surface of solid organs or directly in tissues of interest.

Gastric tonometry, monitoring mucosal PCO_2 and pH, can detect occult hypoperfuson and tissue hypoxia and predicts outcome in critical illness (19). It was enthusiastically introduced in the clinic 20 years ago, but due to a cumbersome methodology and problems with positioning the balloon, digestion of food, levels of gastric acid and bacterial CO_2 production leading to erroneous readings (20), it has not gained widespread use. The tonometer has a gas filled silastic balloon introduced into the gastrointestinal tract, most often in the ventricle. The air inside the balloon equilibrates with the surrounding CO_2 gas so PCO_2 measurements of the sampled air will reflect intramucosal PCO_2 (21). The more recent PCO_2 optode Neurotrend has also been tested in several experimental and clinical settings, but is no longer commercially available.

Sublingual capnography applies an optode for PCO_2 measurement and a temperature sensor placed under the tongue (22). It measures PCO_2 reliably compared to the tonometry method

(23), but was recalled due to serious infections probably caused by bacteria in the solution where the probes were stored. More convenient methods of PCO_2 measuring is warranted, and lately, our group has invented a novel PCO_2 device for tissue monitoring (24;25).

The documentation on intraorgan monitoring of glucose metabolism using the microdialysis system both experimentally and clinically is growing (26;27).

As described above, hypoxic cells become dependent upon the anaerobic breakdown of glucose for production of ATP. This causes an increased conversion of pyruvate to lactate. A lactate/pyruvate (LP) ratio >20 can has thus been used as a marker of ischemia (28). The LP ratio has the advantage of abolishing changes in recovery over the dialysis membrane. Tissue glucose, being the main substrate for energy production, may also provide valuable information. With a reduced blood flow and supply of glucose the tissue levels will rapidly decline.

Glycerol is an integral component of the cell membrane and reflects how severely the cells are deranged. Loss of energy leads to an influx of calcium and activation of phospholipases that split the glycerol backbone from the phospholipids.

Liver transplantation

In Europe more than 5000 liver transplantations are carried out each year. In Norway the transplantation activity is centralized to Oslo University Hospital, Rikshospitalet with approximately 100 transplants a year (10).

In Europe the most common liver diseases leading to transplantation (9) include cirrhosis (58%), (including hepatitis C, alcoholic cirrhosis, autoimmune diseases and primary sclerosing cholangitis (PSC)), cancer 14%, cholestatic disease 10%, acute liver failure 9%, metabolic disorders 6% and others 3%. In the Nordic countries, PSC is the most common

indication for liver transplantation (29). Patients with hepatocellular carcinoma and selected patients with liver metastasis are now more often offered transplantation (30).

The mentioned diseases may lead to complications like variceal bleeding, ascites, hepatorenal syndrome, hepatic encephalopathy, recurrent infections, e. g. spontaneous bacterial peritonitis or bile duct stenosis.

After organ transplantations early detection of compromised organ function is highly needed, both because of the shortage of available organs but also due to the dramatic and not seldom fatal complications (31).

The transplantation procedure

In patients where the clinical evaluation indicates brain death, an angiography will verify lack of cerebral circulation and the patient can then be considered a potential organ donor. Split-liver transplants with are performed in about 20% of transplantations at our hospital, while the use of living donors is not established as a routine.

Pre transplantation blood samples from the donor are analyzed. In contrast to other organ transplantations HLA-typing is not necessary in liver transplantation. The donor is treated to maintain physiological tissue perfusion and optimal organ function.

The harvested liver is stored in a cold preservation fluid (University of Wisconsin solution = UW solution) (32), to slow down the metabolism. The UW-solution contains lactobionate and raffinose to suppress the hypothermia induced cell swelling, potassium to reduce loss from the cells and the antioxidant gluthation.

The liver is removed and replaced by the donor graft with vascular and biliary anastomoses. During the procedure now more often the ”piggy back” method is used where the patients’

caval vein is only partially occluded, and no bypass is needed (33), while in complicated cases continuous veno-venous bypass is still used.

It may take from 6 to 24 hours from the donor liver is harvested until it gets perfused in the recipient. In this period the donor organ is not circulated and receives no oxygen or nutrition. Despite the slow metabolism and low energy requirements following cooling and perfusion with UW solution, cold storage more than 12-15 hours increases the incidence of graft dysfunction significantly (34). From the time the donor liver is removed from the cold preservation and transplanted into the recipient, it is subjected to ”warm ischemia”. In general cold ischemia seems to cause more damage to nonparechymal cells, i.e. Kuppfer and liver sinusoidal endothelial cells (LSEC) and to trigger immunologic activity. Warm ischemia interferes more with the hepatocytes and thereby the metabolic functions (35). The cold and warm ischemia time following a transplantation has a detrimental and synergistic effect on the liver graft (36) so it is important to minimize the duration of both the cold and warm ischemia (34;37).

Complications in liver transplantation

Complications after liver transplantation may be divided into medical, technical, and immunological entities (14).

A compromised preoperative condition, often seen in patients on “urgent call” (acute liver failure) as well as preoperative mechanical ventilation, render the patient more susceptible to complications.

Postoperatively the medical complications include bacterial (pneumonia, cholangitis, sepsis), viral or fungal infections. Toxicity related to immune suppression (nephrotoxicity following treatment a calcineurin-inhibitor or thrombopenia with mychophenolate or rapamycin) are

common complications. Acute kidney injury and renal failure, initially poor functioning grafts or even primary nonfunctioning grafts are other complications during the postoperative course.

The technical complications postoperatively are most often associated with biliary leakage or stricture/stenosis and occur in 11 to 25 % of cases (38). Biliary complications are in general managed endoscopically or by other less invasive methods. Untreated, however, they may lead to graft dysfunction and infections.

Vascular complications are a major threat to the liver graft and must be detected early to avoid graft loss. Hepatic artery thrombosis (HAT) is seen in about 3 – 6 % of the patients, but is more frequent, about 6 - 10% in children. Portal vein thrombosis is seen in about 1 – 3 % of patients (39). HAT has a mortality of approximately 30%, whereas for portal vein thrombosis the mortality is 70% (40). HAT has a mortality of approximately 30%, whereas for portal vein thrombosis the mortality is 70%. Methods for early detection of vascular complications are therefore needed. Postoperative hemorrhage is either due to insufficient surgical hemostasis, bleeding from insufficient vascular anastomosis or coagulopathy.

The immunological complications include acute and chronic rejection. The acute rejection to liver grafts is most often milder and responds better to treatment compared with rejections to kidney and heart grafts (12).

Chronic dysfunction is, however, most often related to recurrence of the primary disease, especially for patients with hepatitis B and C and patients with primary biliary cirrhosis (PBC).

Present perioperative monitoring and management

Patients undergoing a liver transplantation are invasively monitored. The arterial, central venous and pulmonary artery catheters allow continuous measurements of the systemic circulatory status.

Because the patient preoperatively may be critically catabolic and deranged, reestablishing a positive energy balance is crucial. It is essential that the patients get enough carbohydrates the first days so further breakdown of proteins are reduced. After a few days the patient most often can turn to a normal balanced diet. Insulin may be necessary to control the blood sugar levels during the first days after transplantation.

During surgery a transit time ultrasound probe is placed around the portal vein and the hepatic artery to assess hepatic blood flow. Doppler examination is performed once or twice during the next postoperative days, and later on if indicated. It has been demonstrated that hepatic artery flow predicts survival of the liver graft (41). Characteristic changes are revealed by the Doppler examination in hepatic artery thrombosis (HAT) with a high (0.8-0.9) resistive index (RI) and a prolonged systolic acceleration time (SAT). With acute graft rejection there may be a marked decrease in portal blood velocity and an increase in splenic pulsatile index (42-46).

In uncomplicated cases transplanted patients stay one or two days in the ICU, thereafter two weeks on the surgical transplantation ward before transferal to a medical ward. Some patients, however, need a prolonged recovery time either due to preexisting conditions, intraoperative events or postoperative complications (47).

Blood samples are analyzed daily for blood cells, glucose, electrolytes, coagulation status, liver and renal function. Patients with a manifest hepatorenal syndrome or renal failure due to the immune suppressive treatment may need continuous renal replacement therapy (CRRT),

due to overhydration and/or electrolyte disturbances and meticulous monitoring of the fluid balance is mandatory.

Increasing thrombocyte counts indicate adequate liver function while persistent high INR levels may be a sign of a non-functioning graft.

The immune suppressive treatment renders the transplanted patients in danger of opportunistic infections. Screening for cytomegalovirus (CMV) infection is therefore performed every week the first three months. Trimethoprim sulfa is given routinely to protect against pneumocystis carinii, while patients at particular risk are also given antifungal prophylaxis.

Postoperative increases in liver enzymes may indicate cell injury during the preservation period or ongoing liver ischemia (circulatory failure). Increasing values of bilirubin may indicate biliary occlusion or rejection. Ultrasound Doppler, hepatic angiography, endoscopic retrograde cholangiography (ERC) or even surgical intervention may be necessary.

Graft rejection - present diagnostic tools and treatment

Symptoms of rejection may be fever and abdominal pain, and a rise in transaminases are regularly found. A slowly increasing bilirubin may be seen, but the patient may have only a few symptoms. Diagnosis is verified by liver biopsy.

It is important to diagnose rejection as soon as possible to start appropriate anti-rejection therapy. Standard immune suppressive induction therapy starts with a triple combination with corticosteroids, calcineurin inhibitor (CNI) to inhibit the activation of T-lymphocytes and thereby the synthesis of IL-2 and the expression of the IL-2 receptor on the cell membrane and mycophenyl mofetil with effect on both B and T-lymphocytes. Tacrolimus has replaced cyclosporine as the CNI drug of choice. The doses of CNI are kept low because of the danger for renal injury. Patients with, or at risk of developing renal failure, are given delayed start

with CNI and induction treatment with basiliximab, a monoclonal immunoglobulin that binds to activated T-cell receptor for IL-2 is prescribed. This drug combination is used to achieve a strong and immediate effect to prevent acute rejection. The dosages are thereafter gradually reduced as the graft inflammation subsides (48).

When rejection has been verified histologically, treatment with high doses of corticosteroids is started. In some cases the CNI dosages are increased or monoclonal antibody muromonab CD 3 (OKT-3) is initiated that specifically reacts with the T-cell receptor CD-3 complex and thereby blocks the activation of T cells.

The ischemia-reperfusion injury in liver transplantation

Following transplantation with periods of cold and warm ischemia, IRI is inevitable. The graft has prior to transplantation been exposed to an inflammation triggered by the brain death. During the ischemic periods and the following reperfusion the inflammation are further activated.

Several studies have been performed trying to unravel the mechanisms involved in this IR injury (49-61). Liver IRI involves activation and cooperation of both cellular and humoral mechanisms. Main cellular contributors are dendritic cells, $CD4^+$ T-cells, Kuppfer cells, hepatocytes and neutrophil leucocytes (PMN). Humoral substances include the complement proteins, cyto- and chemokines communicating between the cellular components (49). Microvascular endothelial cells are active participants and regulators of the inflammatory process (62;63), and effects on the hepatic microcirculation is essential in IRI (64). The length of the cold ischemic period has been shown to increase cell membrane damage and sensitize the graft for rewarming injury during reperfusion (65). Increased blood levels of liver transaminases (AST/ALT) are used to evaluate the degree of liver injury.

Paradoxically, although reperfusion of the ischemic graft is mandatory for its function and survival and limits the ischemic destruction, it increases the damage caused by the ischemia and causes even more cellular injury.

Injury to the allograft gives production of reactive oxygen species (ROS) which not only cause cellular damage but also release of intracellular agents that act like damage-associated molecular patterns (DAMP)s. The heat shock proteins and mitochondrial DNA, for instance, act as danger signals when getting out of the cell (66) most in the same way as pathogen-associated molecular patterns (PAMPs) such as endotoxin. These substances act as ligands on different receptors and activate the innate immune system via Toll like receptors (TLR4 best described) that is a Pathogen Recognition Receptor (PRR) mainly on dendritic and Kupffer cells and further recruit activated neutrophils and monocytes to the liver (57).

The activation and upregulation of inflammatory mediators may be used diagnostically in estimating the degree of IRI, so far most in experimental settings. Many inflammatory substances like cytokines are expressed in IRI (67).

CXC-chemokines and especially CXCL-10 (IP-10) act as chemoattractants for leucocytes and are produced via TLR-4- IRF-3 (Interferon regulatory factor 3) activation pathway. It plays a role in the immune response of IRI and in an experimental model inhibition reduces the IRI (61;68). The IRI is also a strong inducer of complement activation that leads to production a multitude of inflammatory mediators (69-71).

Rejection of the transplanted liver graft

Although the first liver transplantation with a graft from a non-related donor was performed as early as in 1963, it was not until the discovery of the immune suppressive effect of

cyclosporin (approved for clinical use in 1983) that organ transplantation, including liver transplantation became an established clinical treatment for end stage organ failure.

When transplanting organs between genetically different individuals, a T-cell mediated immune response is initiated which may result in destruction and rejection of the graft (59). To prevent such an outcome the patient receives induction therapy with a combination of immune suppressive drugs that reduces this initial immune response.

Histologically, liver allograft rejection can be divided into three categories (12). In the **hyperacute** rejection (massive hemorrhagic necrosis), there is an anti-donor humoral immune response leading to depositions of antibodies, platelets, fibrin and erythrocytes within the portal venules and hepatic sinusoids. This has been seen after transplantation with preformed antibodies in ABO incompatible combinations although some liver transplantations with A2 donors and 0 recipients and even other ABO incompatible combinations have been successful.

In **acute** rejection (also known as cellular) bile duct inflammation and venular inflammation are seen. Lymphocytes are infiltrating the portal triads. Early acute rejection (earlier than 3 months) responds in general well to increased doses of immune suppressive drugs. Late acute, recurrent and steroid-resistant rejections, however, are more difficult to treat.

Chronic rejection (ductopenic) is characterized by loss of bile ducts, persistent inflammation and arterial foam cell infiltration and is more often associated with graft loss.

A criteria system has been developed to score liver allograft biopsies with acute rejection based on portal inflammation, injury to bile-ducts and degree of inflamed endothelial cells in liver veins. Each category of the rejection injury is graded in a scale from 0-3 giving a rejection activity index (RAI) from 0-9.

Graft versus host disease (GVHD) is a rare complication after liver transplantation. T-lymphocytes from the donor attack organs and tissues in the recipient. With HLA-typing these T-lympocytes can be demonstrated histologically by biopsies from the skin.

An acquired tolerance to the transplanted organ, without life-long use of immune suppression develops in some patients, mostly in children, in particular those transplanted under the age of one year. The microenvironment of the liver can promote tolerance to a much higher degree than other organs. Normally, the immune system and the liver show tolerance to self-antigens. The hepatic sinusoidal system is exposed to food-derived and bacterial antigens, absorbed from the alimentary tract transported to the liver via the portal vein. The exposure normally leads to immunological tolerance rather than immunity of the T-cells to antigens that are not regarded as dangerous to the organism. The liver is thus less prone to rejection and immune mediated injuries than organs like kidney and heart. Acute rejection is influenced by the donor and recipient's antigen composition or degree of foreignness. Donor HLA-antigens are presented to the host T cells. This is done via indirect allo-recognition, where endocytosed material, i.e. HLA-molecules from the transplanted graft, or fragments of these are captured by the recipients antigen presenting cells (APC's) (dendritic cells (DC), Kuppfer cells, sinusoidal cells, and hepatocytes. In direct allorecognition, antigen material is presented to T-lymphocytes via APC from the donor graft. When the activated APCs are presented for the recipients T-cells , activated T cells are "educated" and recruited into the graft which result in tissue damage. The degree of IRI injury described earlier may have an influence on rejection, although an overwhelming IRI may first of all lead to a primary non-fuctioning or poorly functioning graft. Central to the inflammatory response is further promotion of reactive oxygen species (ROS) and stimulation of the innate immune system and maturing of dendritic (DCs) and other immunological active cells, leading to increased production of inflammatory

mediators and activation of the adaptive immune system which, via $CD4^+$ T-cells lead to the allograft rejection (53;56).

Immune suppressive treatment has serious long-term side-effects including development of posttransplant lymphoproliferative disease (72) and cancer. Earlier, episodes of acute rejection were treated with more aggressive immune suppression, a treatment which later was associated with chronic rejection (12). Aggressive immune suppressive treatment targeted not only the cytotoxic T-cells, but also the regulatory T-cells (Tregs, which actually are T cells expressing both $CD4^+$ $CD25^+$) that probably play a crucial role in development of tolerance (59;73). In the "decision" between tolerance or immunity and rejection, different cytokines modulate alloresponsive T-lymhocytes to either develop a proinflammatory response ending in graft destruction or a tolerogenic response resulting in graft acceptance (74).

Today, there is a tendency towards less aggressive immune suppressive regimes. Although this may lead to more episodes of acute rejection, the incidence of late rejections and graft loss has decreased. With milder immune suppression regimes, recruitment of and generation of Tregs will develop as part of an acute rejection reaction, leading to a better tolerance to the graft. In fact, it has been shown that some patients can manage without any immune suppression at all (75). Strategies to predict which patients do not need such treatment, is a challenging task. During acute rejection in liver transplant patients it has been demonstrated decreased levels of circulating $CD4^+$_$CD25^+$ Tregs (76). The inflammatory donor mediator IP-10 and recipient mediator CXCR-3 have shown to play a role in initiating acute rejection in a heart transplantation model (77-79). IP-10 has also been shown to predict fibrosis progression after liver transplantation for Hepatitis C infection (77). Increased complement activation is documented in liver transplant rejection (80-83), and has been associated with poor graft functioning (84). IL-2 is produced during rejection (85) and has been used as a

response measure for the anti-rejection treatment. Elevated levels of IL-8 in biopsies (86) and in blood (87;88) have also been reported during rejection.

Aims of the work

The main aim of our research group has for several years been developing and evaluating implantable microdevices for detection of ischemia, both in experimental animal models and clinically. Study 1 in this thesis is a study with this aim. In an experimental porcine model we conducted a study with a progressive hemorrhagic model to compare different methods of ischemia detection, to characterize the effect of ischemia on different organ systems (liver, kidney, intestine and skeletal muscle) and to define the transition from aerobic to anaerobic metabolism.

Through this hemorrhagic study we explicitly gained experience with the microdialysis system as a reliable method to monitor and describe renal, hepatic and intestinal and muscular glucose metabolism under ischemia with the intermediate metabolic parameters glucose, pyruvate, lactate, LP ratio as well as glycerol. Based on this work, where we reliably detected ischemia in liver, we wanted to investigate whether microdialysis could be used to detect ischemia in a clinical setting, namely in monitoring transplanted livers in the immediate postoperative period (first week). Since there is a clinical need to detect ischemia caused by hepatic artery or portal vein obstruction as soon as possible to save the graft, we learned from the animal study that ischemia was reliably detected within 1 hour so implementing hourly measurements would detect ischemia earlier than current methods.

Microdialysis in human liver transplantation had been done in pilot studies for metabolic monitoring (89;90). In addition to monitoring ischemia we wanted to explore whether the microdialysis system also could be used to monitor immunological inflammatory parameters (cytokines and complement activation). In a methodological in vitro study we therefore

explored the ability of the microdialysis systems to recover relevant immunologic inflammatory parameters using a microdialysis catheter with a 100 kD pore size. The microdialysis system in transplanted liver grafts for monitoring cytokines and complement activation had not been tested in clinical studies. Based on the experience with microdialysis in the experimental studies (paper I and II) with metabolic intermediates and inflammatory mediators we initiated a study on liver transplanted patients, expanding the use of microdialysis to also include monitoring of immunological inflammatory parameters in addition to metabolic parameters. We wanted, by way of selected inflammatory and metabolic parameters to characterize the postoperative course in patients without complications and to explore whether ischemia or transplant rejection could be detected, and if that was the case, would complications be detected earlier with microdialysis than with standard monitoring. The results could have implications on how we routinely monitor liver transplanted patients.

Paper I

In this experimental porcine study on hemorrhagic shock, we aimed to:

- Characterize hemodynamic responses in different organ systems when cardiac index is reduced by hemorrhage.
- Study gas tensions, acid base variables and metabolic intermediates in skeletal muscle, kidney, liver and intestine during progressive hemmorhage.
- Compare PCO_2 to metabolic parameters of ischemia for their ability to detect hypoperfusion and ischemia.

Paper II

In this in vitro study we aimed to:

- Determine the in vitro recovery of cytokines like TNF-α, IL-1b, IL-6 and IL-10, the chemokines IL-8, MCP-1, IP-10 and MIG and the complement anaphylatoxins C3a, C4a and C5a, using reference preparations with known concentrations of the analytes.

- Compare recovery rates through microdialysis membranes with two different molecular weight cut offs (20 kDa and 100 kDa) at different flow rates (0.3,1 and 5 μl/minute).

Paper III

In this clinical study where microdialysis catheters were inserted in the liver and subcutaneously peroperatively, and kept in place for 7 days, we aimed to:

- Characterize the pattern of lactate, pyruvate, glucose and glycerol and relevant inflammatory substances like cytokines and parameters of complement activation in patients with a normal postoperative recovery

- Explore whether microdialysis with a 100 kDa cut off filter could be used to detect liver ischemia, rejection or other complications after allograft liver transplantations.

Materials and methods

Biosensors for tissue gas monitoring

Three different biosensors were used: An electrochemical sensor (MI 720 Microelectrodes, Bedford, NH, USA) measuring PCO_2, a Clark-type sensor for oxygen tension measurements (Licox™) and a multisensor device, Neurotrend™, using optical sensors for the measurement of pH, PCO_2, and pO_2.

PCO_2 measurements based on a modified electrometric pH electrode

The Severinghaus sensor is a modified electrometric pH electrode measuring tissue PCO_2 (91) It consists of a glass pH sensor within a chamber which at the tip is covered with a Teflon membrane permeable to gases including CO_2. In the space between the glass surface and the membrane there is an aqueous medium of 5 mM bicarbonate buffer.

Through the gas permeable Teflon membrane CO_2 enter the bicarbonate solution. The following change in pH is detected by the pH sensor. PCO_2 is calculated according to the linear relationship between log PCO_2 and pH.

The Severinghaus PCO_2 sensors were calibrated by a three point calibration with PCO_2 tensions from 3 to 25 kPa. The values were checked by a blood gas analyzer just before and just after the experiment and drift was corrected.

The sensors were inserted into the intestinal lumen or placed on the muscular surface. To avoid injury to the muscular tissue and prevent gas evaporation the electrodes were placed in rubber gaskets with the tip placed 0.5 mm from the surface.

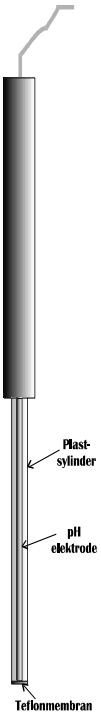


Figure5: The Severinghaus –type sensor

Multiparameter sensor (Neurotrend™)

The Neurotrend™ sensor is a disposable, single-use device for the continuous measurement of tissue pH, PCO_2, PO_2, and temperature. While the pH and PCO_2 sensors work on the principle of optical absorption, the pO_2 sensor apply the principle of fluorescence quenching, whereby the intensity of a fluorescent optical emission from an indicator is reduced in the presence of oxygen. The diameter of the probe is 0.5 mm and the four sensing components are

located at different intervals along the distal 4 cm of the polyethylene probe which is permeable for O_2 and CO_2. The outer surface of the probe is coated with covalently bonded heparin to prevent fibrin deposition. The flexible wire part of the device has a length of 600 mm. The Neurotrend sensors were calibrated before use in a separate calibration unit (Diametrics Medical). This unit is also used for data capturing and storage. Values of pH, PCO_2, pO_2, and temperature were recorded every 60 second.

Clark-type sensor for Oxygen Tension Measurements (Licox™)

To monitor oxygen tension levels directly we used a miniaturized Clark-type sensor which can be inserted into the tissue (Licox, Integra, Germany). The sensor is calibrated with a one point calibration in air before use. This sensor is 0.7 mm in diameter and contains a silver anode and a platinum cathode which is covered by a membrane that is oxygen-permeable. A constant voltage is applied to create a reaction between oxygen and the platinum electrode. The drained current between the two electrodes is proportional to the tension of the oxygen in the tissue.

Animals used in study I

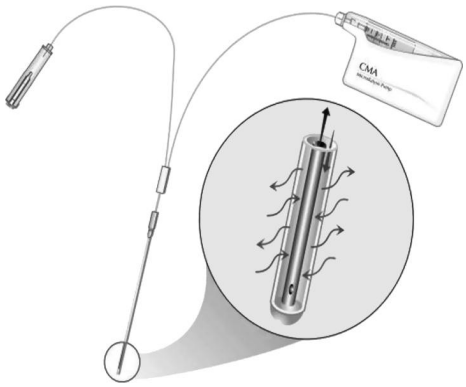
Before the commencement of this study, we discussed carefully if we should include a control group and decided not to do so for the following reasons: i) The animals in this study are their own controls as the parameters during blood loss are compared to baseline values. Six consecutive baseline values were collected during 90 min after surgery, and the animals were very stable; ii) we have carried out several studies on models very similar to this one where the animals have undergone major abdominal surgery receiving exactly the same anesthesia

as in this study and been anesthetized for up to 12 hours after surgery. These animals have been very stable and tolerated the procedure without significant changes in organ blood flow and have revealed no signs of ischemia. We are therefore convinced that findings in this study are due to the bleeding protocol and not to surgery and anesthesia per se; iii) in our institution we carry out experiments on more than 200 pigs a year, and we are striving to limit the number of animals used as much as possible (both for ethical and economic reasons), and we therefore usually only include control animals in the pilot phase or in new types of procedures. As the procedure used in this study is nearly equivalent to other studies carried out, we reasoned that a control group was not deemed necessary; iv) we know from previous studies that the drift of microdialysis, Neurotrend and Licox are minimal and cannot account for the changing values over time. The drift in the Severinghaus-type of sensors was taken care of by careful calibration with known PCO_2 values before and after the experiment and the drift was mathematically corrected.

Microdialysis

Microdialysis is a method for tissue sampling of small molecules based on a passive diffusion of substances across a semipermeable hollow fiber dialysis membrane. The microdialysis probe is built up as a concentric tube where the perfusion fluid flows through an inner tube to the distal end where the direction is reversed. The solution enters the space between the inner tube and the dialysis membrane where the exchange of molecules takes place. The difference in concentration over the membrane governs the direction of the gradient. A 2.5 ml syringe with a perfusion fluid is placed in a portable battery driven pump adjustable for different flow rates (0.1–5 μl/min) and connected to the microdialysis catheter. Two different microdialysis catheters were used: the CMA 61 20 kDa and CMA 71 100 kDa. The microdialysis catheters were inserted into the tissues by a splitable introducer. When starting the battery driven pump,

Figure 4: The Microdialysis system. Fluid in the syringe is pumped into the microdialysis catheter entering the space between the outer and the inner concentric tubes. Exchange of substances takes place through the outer membrane. The dialysate then enters the inner tube at its distal end and is propulsed into a microvial at the end. The accumulated fluid in the microvial can then be analysed (figure used with permission from Microdialysis, Stockholm, Sweden).



the perfusion fluid is driven through the catheter. The solution then equilibrates with the surrounding extracellular tissue fluid over the microdialysis membrane. The dialysate is collected in microvials connected to the catheter.

Application of microdialysis in studies II and III

In paper II we investigated the ability of microdialysis membranes with 20 kDa and 100 kDa to recover cytokines, chemokines and complement activation factors. Reference preparations for cytokines and chemokines were prepared from plasma after incubation of human blood with lipopolysaccharide (LPS) and for anaphylatoxins by incubating human plasma with heat aggregated immunoglobulin G (HAIGG).

Microdialysis was performed in these preparations with known concentrations of the relevant substances with the two different membranes at three different velocities (0.3, 1 and 5 µl/min). The mediators were then analyzed with cytometric bead assays.

In paper III microdialysis catheters were used to monitor immunological mediators in transplanted livers. Microdialysis catheters with 100 kDa MWCO were inserted into the left

and right liver lobe respectively via a split device, tunneled out through the abdominal wall and connected to a CMA107 microdialysis pump. A reference catheter was inserted subcutaneously in the lower pectoral area.

The metabolic parameters glucose, lactate, glycerol and pyruvate, and the calculated ratio between lactate and pyruvate, LP ratio were analyzed by the CMA106 Analyzer every hour the first 24 hours, thereafter every two hours the next six days. Samples for inflammatory parameters were frozen every 4 hours at -70 ºC and analyzed later with the bead assay method.

During removal a small part at the distal end of one the hepatic catheters was left in the abdomen as it broke when trying to pull it out. We did not regard it possible or necessary to remove this small surgically, which was explained to the patient. The patient did not experience of any symptoms or signs related to this foreign body. There were reported similar problems in other studies, and the manufacturer withdrew the catheters from the market in order to make them more robust. Due to a halt in the production of catheters which lasted more than 6 months, we ended inclusion of patients after 20 cases. More than 200 of the new catheter have been inserted in the liver at our hospital. None of these have broken during removal.

There were no other complications, for instance bleeding or infection related to insertion and use of the catheters. In two cases where patients were reoperated the first postoperative day, the catheters were removed and not replaced. In three other patients one of the hepatic catheters stopped to function after 2, 3 and 4 days respectively, but since we had one catheter in each lobe we were still able to collect data.

Analysis of immunologic parameters recovered through microdialysis

Both in study II and III cytokines and complement activation factors were analyzed using cytometric bead assays. In the cytometric bead assay method, beads, each coated with a unique cytokine, chemokine or anaphylatoxin detection antibody directed against the respective analytes are mixed with the analyte samples. The particles are dyed with different fluorescence intensities, separately detected by flow cytometry. The different capture beads bind their specific analytes which is then detected by specific antibodies conjugated with phycoerythrin present in the buffer solution.

In study III we used the Bio-Plex Human Cytokine27-Plex Panel kit (Bio-Rad Laboratories Inc., Hercules, CA). Here the beads are also coated with cytokine antibodies. When the beads are incubated with the samples, a secondary biotin-conjugated antibody is added and then a reporter molecule (streptadivin-PE) binds to the biotin. The samples are then further run in a modified flow cytometer with two lasers, one which excites the beads and one which excites the reporter molecule. In this way, the beads are "separated", and the amount of each of the cytokines can be quantified.

Clinical study (Paper III)

Patient characteristics

An important aim of the clinical liver transplantation study was to describe the development of metabolic and immunologic parameters both in normal and complicated complicated cases. There were 14 patients without major clinical complications during the time the catheters were inserted. However, several of these developed complications after removal of the catheters as shown in the table below. The following patients were excluded from the normal

group: Four patients who underwent rejection during the first nine days (one of them also a HAT), one patient an ascending bile-infection and one patient with a non-functioning microdialysis catheter. Thus, six patients were excluded from the normal group of fourteen. Both retransplants were in the group of excluded patients. The fourteen normal patients were also used as the normal population for immunological studies. However we also analyzed the three patients described in the paper as patients with complication (case 1 – 3) in the same kit batch in order to have comparable results, i. e. avoid batch variations. Since each kit has 80 wells, we had to exclude 3 normal patients to get space for the complicated ones. We could have used another kit for these three patients, but that would give batch variations, and would considerably increase the cost. We therefore decided that 11 patients would suffice to get data for normal patients.

As demonstrated in Table 1 below, only 5 out of the 20 patients were actually without any registered events or postoperative complications, but the majority of these complications occurred after the microdialysis catheters were removed.

Patient	**Diagnosis**	**Events/complications**
1	Hyperoxaluria	Combined liver/kidney tx, rejection of kidney
2	Cirrhosis, alcoholic	Ascending bile duct infection
3	PSC	Biliary leakage
4	Acute viral hepatitis	Multiple complication including rejection in retransplanted graft, patient died after 6 months (Case 2 in paper III)
5	PSC	Recurrence, retransplanted x 2
6	Cirrhosis, Hepatitis C	Recurrence of Hepatitis C

7	Bile duct atresia	Initial high resistance in hepatic artery
8	Cirrhosis, alcoholic Hepatocellular Carcinoma	ALT 4445 IU the first day
9	Cirrhosis	Postoperative lung edema
10	Cirrhosis	None
11	Portal vein trombosis	HAT, rejection day 11, catheter 6 days (Case 3 in paper III)
12	Cirrhosis, alcoholic	None
13	Cirrhosis, unspecific	Catheter broke during removal
14	Steathosis	None
15	PSC	Recurrence
16	Hemangioendothelioma	Rejection verified day 7, catheter 11 days (Case 1 in paper III)
17	PSC	None
18	PSC	None
19	Budd Chiari disease	Stenosis of choledochus, rejection verified day 9, catheter removed after 5 days
20	Autoimmune hepatitis	Aspergillus infection, patient died after 5 months

Table 1. Complications after liver transplantation for patients in study III

Statistics

In study I, baseline values of the variables served as controls. To reduce the effect of transient flow variations we calculated replicates as the mean of the two former, the present and the two next flow measurements.

For each ischemia parameter in each organ a critical cardiac index value, CI_{crit}, was determined by dual line regression analysis (92).

All variables are presented as median with interquartile range (25% - 75%). Areas under the flow curves (AUC) were calculated using the sum of rectangles method. To determine significant differences between the critical values, the non-parametric methods Kruskal-Wallis One Way Analysis of Variance on Ranks and Post-hoc Student-Neuman Keuls (SNK) test for a multiple comparison of all the groups were used.

Time derivates of PCO_2 and lactate, $dPCO_2/dT$(time) and d[lactate]/dT for the investigated organs were calculated to show differences in the rate of increase under aerobic and anaerobic conditions. The Kruskal-Wallis One Way Analysis of Variance on Ranks and Post-hoc Student-Neuman Keuls (SNK) test for a multiple comparison of all the groups were used

In study III data are shown as median with 25 and 75 quartiles. We used a Wilcoxon rank sum test to decide differences between values from liver and subcutaneous tissue (in paper III it erroneously says Wilcoxon signed rank test). Bonferroni corrections were carried out. AUC (Area under the curve) was used to compare the metabolic variables in the liver and subcutaneous tissue.

Summary of results

Paper I:

Tissue gas tensions and tissue metabolites for detection of organ hypoperfusion and ischemia

Lars Wælgaard, Berit Marie Dahl, Gunnvald Kvarstein and Tor Inge Tønnessen

During continuous hemorrhage we observed an almost linear, inverse relation between decreasing cardiac index (CI) and increasing blood loss. There was a more pronounced decrease in blood flow to kidney and muscle than reduction of CI. Flow in hepatic artery decreased to about the same degree as CI while intestinal blood flow, measured as portal flow, fell relatively less compared to CI. From baseline to a CI of ~3500 ml/kg/m^2 we observed only a moderate fall in mean arterial pressure (MAP), but thereafter a more rapid decline was noted.

During reduced blood flow, but still aerobic conditions, PCO_2 increased significantly (1-4 kPa) in all the investigated organs There were no significant change for tissue metabolic parameters lactate, LP ratio, and glycerol .

Until a reduction in CI to about 3000 mL/kg/m^2 PO_2 fell at a rate of 0.4-0.5 kPa/L decline of CI. After this a more pronounced decrease of 1.3-1.8 kPa/L was seen. The kidney reached zero PO_2 significantly earlier than in the other organs.

Below the critical CI levels LP ratio, lactate and glycerol increased significantly, while PCO_2 attained a much faster increase than during aerobic conditions. Thus, only PCO_2 increased significantly both during aerobic and anaerobic conditions.

In kidney and liver there were no significant differences in CI_{crit} for PCO_2, lactate, pH and bicarbonate. In muscle and intestine we found a slightly lower CI_{crit} for lactate than for PCO_2.

In all the investigated organs CI_{crit} values were lower for glycerol and LP ratio than for PCO_2 and lactate. There were no significant organ differences for glycerol and LP ratio.

In the intestine the microdialysis catheters were placed intraluminally, and pyruvate and glucose were thus not detected until the mucosa had become leaky, and the barrier seriously disturbed (93;94). Under baseline conditions glucose is not detectable with microdialysis in the gut lumen (95). The basal intraluminal values for lactate and glycerol were almost identical to what other researchers have found (0,08 vs 0,11 mmol/l and 15.9 vs 16.2 µmol/l respectively) (96). In the intestine we did not calculate the LP ratio as several values of pyruvate was below the detectable level. Glucose in liver and kidney increased during the first period of hemorrhage, but dropped sharply at the anaerobic threshold. Mean arterial hemoglobin stayed unchanged until about 60 % of total blood loss, and then it fell from about 9.5 g/dL to 6 g/dL at the time of death.

Paper II:

Microdialysis for Monitoring Inflammation: Efficient Recovery of Cytokines and Anaphylotoxins Provided Optimal Catheter Pore Size and Fluid Velocity Conditions

L. Wælgaard, A. Pharo, T. I. Tønnessen, and T. E. Mollnes.

Microdialysis has become an established method to evaluate tissue metabolism, and in the clinic, measuring low molecular weight metabolites like glucose, lactate, pyruvate and glycerol can give valuable information to diagnose tissue ischemia by use of a 20 kDa molecular weight cut off membrane.

With the introduction of a 100 kDa microdialysis catheter measurements of inflammmatory mediators have become possible. Because no systematic studies had been done on

determining the recovery of a broader panel of inflammatory mediators like cytokines and anaphylatoxins, we wanted to evaluate the microdialysis system for this purpose.

In an in vitro study, we compared the use of 20 kDa and 100 kDa microdialysis catheters at three different velocities (0.3, 1.0 and 5.0 µl/min) in recovering relevant inflammation markers from reference preparations, namely the cytokines TNF-α, IL-1β, IL-6 and IL-10 (17–28 kDa); and the chemokines IL-8, MCP-1, IP-10 and MIG (7–11 kDa); in addition to complement anaphylatoxins (C3a, C4a, C5a; 9–11 kDa). Recovery with the 100 kDa filter was as follows: IL-1β 75%, MCP-1 55%, MIG 50%, IL-8 38%, C4a 28%, IP-10 22%, C5a 20%, C3a 16%, IL-6 11%, IL-10 8% and TNF-a 4%. The highest recovery for the chemokines and anaphylatoxins was consistently at velocity 1.0µl/min, whereas IL-1β and IL-10 recovered most efficiently at 0.3 µl /min.

Paper III:

Microdialysis Monitoring of Liver Grafts by Metabolic Parameters, Cytokine Production, and Complement Activation

Lars Wælgaard, Ebbe Billmann Thorgersen, Pål-Dag Line, Aksel Foss, Tom Eirik Mollnes and Tor Inge Tønnessen.

We wanted to explore whether microdialysis with a 100 kDa cut off filter could be used to monitor ischemia, inflammation and rejection after liver transplantation. Catheters were inserted into both the left and right liver lobe. A reference catheter was introduced subcutaneously more than 5 cm away from the surgical wound. The catheters were left in place for a median of 156 hours (138.25-167.5). Metabolic parameters were analyzed bedside with the CMA 106 Analyzer at 1 hour intervals the first 24 hours and then every second hour for the next six days.

In patients with an uncomplicated postoperative course, lactate in the liver fell to normal values within 12 to 24 hours. Also in the subcutaneous tissue a decrease to low values was seen, but a higher steady-state value was measured in liver. The LP ratio also showed a rapid decrease to a steady level of about 10 in the liver, bur was significantly higher in the subcutaneous tissue. Glycerol was significantly higher in subcutaneous tissue than in liver. In patients undergoing an uneventful postoperative course, IL-6, IL-8 and C5a stayed low, but median IP-10 increased from 200 to 3000 pg/mL in the liver.

Three of the four patients with biopsy verified rejections are described in detail in paper III (figures 3-5).

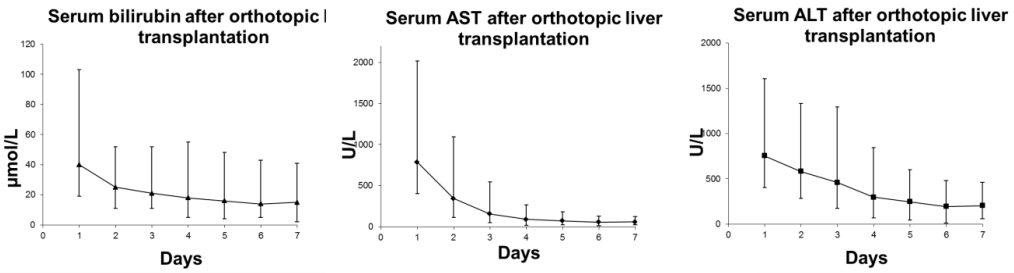
Case 1 had a normal decrease in lactate reaching normal values within 24 hours postoperatively. On days 3 - 6 lactate increased in the liver and peaked at a value of 5 mM, while there was no increase in lactate in the SC catheter or in the blood. There was no increase in LP ratio or glycerol, ruling out ischemia as a cause of the high lactate. Pyruvate rose to the same extent as lactate, which is compatible with a hypermetabolic state. Complement C5a was measured to 8000 pg/mL immediately postoperatively, but decreased the next two days. Concomitantly with the rise in lactate, a pronounced increase of IP-10, IL-8 and C5a in liver was found. There was no increase in ALT in blood until day 7 when a biopsy verified acute rejection. On day 7 high dose steroid was given as anti-rejection treatment and lactate, pyruvate, IP-10, IL-8 and C5a promptly declined to normal values strongly indicating that these parameters detected rejection 4 days earlier than rise in ALT, and anti-rejection treatment reestablished normal values.

Case 2: Patient with fulminant hepatitis. This patient had a very complicated course as described in paper III. Four days after retransplantation C5a and IL-8 increased to 2500 and 4000 pg/mL, respectively. Concurrently, IP-10 increased to 2200 on day 3. Histological examination verified rejection on day 9, four days after the catheters were removed.

Case 3: High values of both liver glycerol and liver enzymes that did not normalize postoperatively. IL-8 was elevated from the start of the postoperative course and fell gradually to values 2-3 times higher than those without major complications. At day 7 right hepatic artery showed reduced flow at the same time as LP ratio and glycerol suddenly peaked in the right lobe indicating ischemia. On day 13 the patient was rescheduled for surgery due to a choledochus stenosis. Prior to this, a new Doppler revealed marginal flow in the right hepatic artery, and a thrombus was removed at surgery. In the second postoperative course with microdialysis reestablished a rapid increase of IP-10 was observed reaching 13000 picog/mL. A week later a histological examination verified acute rejection.

The fourth patient with biopsy verified rejection showed normal metabolic values and low IP-10 in the microdialysis catheters that was removed on day 5. Due to S-bilirubin elevation on day 5 and a suspected stenosis in ductus choledochus the patient was endoscopically treated with implantation of a biliary stent. Nevertheless, bilirubin continued to increase. A histological examination verified rejection on day 12.

For all the transplanted patients median duration of the total ischemia time ((time from clamping of aorta to restituted flow in the portal vein) was 9.5 (8-11) hours. Median initial flow was 300 (233-429) ml in the hepatic artery and 1326 (1121-1925) ml in the portal vein.



In the figure above AST, ALT and bilirubin in serum from the 20 patients in study III undergoing orthotopic liver transplantation (OLT) is shown. Median values were normalized

witin one week, but the variance is large showing that several patients had higher than normal values. Three of the patients had values of AST and ALT > 2000 U/L in the first postoperative days indicating a severe ischemia-reperfusion injury.

Doppler ultrasound was performed in all the transplanted patients before the abdomen was closed, and then at least once daily. In three patients Doppler ultrasound revealed decreasing blood flow (resistive index > 0.9) and increasing lactate and LP ratio consistent with mild ischemia. (one of these patients is described as case 3). In the two other patients Doppler ultrasound revealed high flow resistance, and from day 1 to 2 they developed elevated LP ratios from 11 to 19 and 12 to 31, respectively. Later hepatic artery flow and the LP ratios rapidly normalized.

Three other patients showed a high resistive index (RI) of 0.8-0.9 (normal values 0.6-0.75) which normalized to about 0.6 on the second or third day, without any elevation in the LP ratios.

Discussion

Changes in regional and systemic blood flow during hemorrhagic shock

We applied a continuous volume-controlled hemorrhagic shock model where blood was withdrawn continuously equivalent with 8 % of the total blood volume every hour. In a previous study we used a stepwise bleeding pattern (97). Continuous bleeding, rather than stepwise, is often encountered in patients with major occult bleeding from solid abdominal organs. We believe our model is highly relevant for such clinical situations. In most other studies shock has been achieved by rapid bleeding to a given blood pressure (23;98;99) by abruptly drawing a specific percentage of the blood volume (5;100) or until the subjects get unstable (18). These studies have correspondingly not been able to define the exact onset of organ ischemia (anaerobic threshold). This is the first study on hemorrhagic shock with simultaneous measurements of circulatory and multiple parameters in such a large number of organs.

With increasing hemorrhage we observed an inverse relation between hemorrhage and CI. Muscular blood flow declined significantly and more than systemic blood flow. Blood supply to the kidney declined earlier and reached zero flow earlier than in the other organs. Intestinal blood flow was relatively better preserved than CI while hepatic flow had a parallel decrease compared with CI.

From a 40 to 50% reduction in CI, flow to the kidney was reduced from 50 to 90%. Another study similarly measured an abrupt reduction in renal flow at about 40% blood loss (101). Under non-anaesthetic conditions a renal vasodilatation may take place during early stages of hemorrhage, while in anesthetized animals vasoconstriction has been reported (7). Under physiological conditions the kidney uses 90 % of its energy on fluid and electrolyte regulation and tubular reabsorption. The blood and oxygen supply is thus much higher than what is needed for basal metabolism, and renal oxygen consumption increases and declines

proportionally with blood flow and oxygen supply. So despite the early and more pronounced drop in flow to the kidney, signs of anaerobiosis were not seen earlier, because VO_2 in the kidney was reduced much more than in other organs before turning to anaerobic metabolism. Hepatic artery flow decreased to about the same degree as CI, but remained almost unchanged between 30 and 50 % hemorrhage. This is in agreement with the unique buffer response of the hepatic artery, where the hepatic artery can dilate when portal flow is restricted, and can increase its flow by more than 25 % (97).

In our study intestinal blood flow, indirectly measured by flow in the portal vein, was significantly better preserved than CI ($p<0,05$) This is in agreement with what we found in a previous study (7). Again there seems to be a difference in vascular tonus in anesthetized and not anesthetized animals with higher splanchnic flow in anesthetized cases (7). However, even though intestinal blood flow was better preserved, signs of intestinal hypoxia appeared earlier than in the other tissues. Like previous studies we found indications of intestinal ischemia at blood flow levels 50-60% of baseline (102).

The anatomically close proximity of the arterioles and venules in the intestinal mucosa and gradient between arterial and venous oxygen make O_2 to shunt at the lower part of the villi. Thus, at the top of the villi PO2 is low rendering the villi very prone to ischemia. Due to shunting and low oxygen tension, CO_2 accumulate in the tip of mucosal villi. This counter-current shunting of oxygen makes the intestinal mucosa more vulnerable to hypoxia following hypovolemia (103).

Hemoglobin was stable until CI of about 2000 mL/kg/m^2, and then it decreased from about 9.5 to 6 at the time of death. Since no external fluid was administered to the animal, the decrease in hemoglobin is highly likely recruitment of interstitial fluid into the circulation as an attempt to counteract the development of shock due to blood loss.

Metabolism in hemorrhagic shock

As PO_2 approached zero in the four organ systems, we observed a rapid increase in lactate and LP ratio and a steep fall in pH and bicarbonate. At this point we also observed a marked fall in glucose and increase in glycerol and PCO_2 (2-4 fold) in the four organ systems. Though lactate is most often regarded as a marker of ischemia, increasing tissue levels of lactate may also reflect hypermetabolism (104), inhibition of pyruvate dehydrogenase (105) and inhibition of mitochondrial respiration (106;107). The LP ratio is not changed in hypermetabolic conditions because a parallel increase in lactate and pyruvate keeps the LP ratio unchanged.

In subcutaneous tissue adrenergic substances may stimulate phospholipase A to split the phospholipids into glycerol and fatty acids. During the immediate postoperative phase glycereol will therefore increase subcutaneously. This moderate rise does not indicate ischemia (108). High glycerol values measured in organs after transplantation, on the other hand, is most likely a sign of cell membrane disintegration and damage.

During anaerobic conditions protons are released from lactic acid, hydrolysis of high energy phosphate compounds (ATP and ADP) and dissociation of phospholipids. A pronounced buffering of H^+ ions by proteins and HCO_3^-, however, prevents a life threatening acidosis. As much as 30-50% of lactic acid is buffered by HCO_3^-. Consequently, lactate and PCO_2 increase in parallel during ischemia (102). In this buffering process, PCO_2 rises at the expense of HCO_3^-, so the total CO_2 content is not changed.

The role of PCO_2 as a parameter of ischemia

There is a large body of evidence from experimental studies with tonometric measurements in the stomach or in intestine supporting the use of tissue PCO_2 as a marker of ischemia of (21;109-112). It is well established that PCO_2 detects regional hypoperfusion and flow stagnation (113). However, controversy exists whether PCO_2 is a sensitive and specific

detector of anaerobic metabolism. Some authors consider tissue PCO_2 a universal marker of tissue hypoxia (17;114), others argue that the increased tissue levels following ischemia reflects the reduced flow per see and not increased anaerobic production (115-119). This might look like a controversy, but all authors find that PCO_2 increases with tissue hypoxia caused by ischemia. In rare cases of pronounced anemia or extreme systemic hypoxia tissue hypoxia may occur with less increase in PCO_2 than if tissue ischemia is caused by ischemia. In these cases PCO_2 is generated under ischemic conditions, but due to high compensatory blood flow CO_2 is effectively removed from ischemic tissue, making the rise in PCO_2 less pronounced. Some researchers have proposed that the anaerobic threshold can be defined as the point where the gradient between tissue and venous PCO_2 starts to increase (118;119). The gradient between tissue and venous gas tensions started to rise concurrently with PO_2 approaching zero. Thus, the steep rise in tissue PCO_2 seems to start when the anaerobic threshold is reached.

The most important finding in our study is that PCO_2 is a marker of hypoperfusion both under aerobic and anaerobic conditions. PCO_2 increased significantly by 1-4 kPa also during initial hemorrhage when the tissues was still able to compensate the reduced blood supply by increasing their oxygen extraction and maintain aerobic metabolism. This is different from the other parameters investigated which did not change significantly under aerobic conditions. PCO_2, however, heralded anaerobic metabolism simultaneously with lactate and L/P-ratio when measuring in kidney and liver, but earlier in intestine and skeletal muscle. It must be emphasized that despite significant differences in CI_{cri} , the differences for various ischemia markers are small.

In study I we found anaerobic metabolism in abdominal organs when arterial values for pH, bicarbonate, BE and lactate were still normal. Values of systemic parameters reached significance at lower CI-values (1900-2000 $mL/kg/m^2$) than in abdominal organs. Thus,

anaerobic metabolism in abdominal organs may be present despite normal arterial levels of these variables.

Recovery of substances across the microdialysis membrane

Microdialysis is a reliable method for sampling small hydrophilic molecules. With long enough membrane, slow speed and small hydrophilic molecules like glucose, pyruvate and lactate, the recovery comes close to 100 % (120). The relative recovery is defined as the ratio between the concentration of the actual substance in the dialysate and in the tissue, and is expressed as percentages.

For larger molecules like cytokines, the recoveries may be as low as a few percent even when the pore size of the dialysis membrane is 100 kDa (121).

Several factors will influence the degree of recovery like analyte concentration, diffusion coefficient which includes physical and chemical properties of the molecule, the molecular quaternary structure and the degree of hydrophilicity and hydrophobicity. While small hydrophilic substances easily pass the dialysis filter, hydrophobic, large proteins may stick to the microdialysis membrane, clearly influencing the recovery. Finally, perfusion flow velocity, probe membrane properties, temperature and resistance to diffusion of the analytes will also influence the recovery within different types of tissues (121).

Significantly more protein is recovered with the 100 kDa catheter (122). Helmy et al. (123) found that nine out of 12 cytokines showed improved relative recovery when a colloid (perfusion fluid supplemented with 3.5% human serum albumin) was used as the perfusate compared with a standard crystalloid fluid. Relative recovery for the two perfusion fluids was 145% and 92 %, respectively. Addition of a colloid such as dextran or albumin to the perfusate (in our studies Plasmodex as recommended by the CMA) reduces the loss of fluid across the microdialysis membrane, which otherwise would lower the relative recovery.

The concentration of inflammatory mediators is very low under physiological conditions, at nano- and even picogram levels. During inflammation, however, the levels may increase 10 to 1000 fold so detecting changes will then be sufficient for monitoring.

In vitro calibration can be done with mediums containing known concentrations. In clinical trials, however, it will not be possible to calibrate with known reference values (124), Investigators have compared in vitro and in vivo measurements with a similar microdialysis probes (125-128). In vivo recovery tends to be lower than the in vitro levels (129). Thus, one must regard the recovery numbers as semiquantitive. Systematic studies on recovery of inflammatory substances in vivo are lacking.

In vivo, microdialysis IL-6 and IL-1β from human brain has shown to recover with 100 kDa pores (130). In a clinical comparison of catheters with 20kDa and 100 kDa molecular cut off small cerebral metabolites (lactate, pyruvate) recovered comparably well with a cut off of 100 kDa and 20 kDa. The 100 kDa catheters can therefore be used for measurements of small and large molecules (122).

Microdialysis as a diagnostic tool in liver transplantation

In a feasibility study (paper III) we used microdialysis catheters in the liver and subcutaneous tissue to monitor patients the first seven days after liver transplantation. We were able to confirm that it is possible to recover both small metabolic and large inflammatory molecules from transplanted livers by the use of microdialysis catheters with 100 kDa MWCO.

Regarding the metabolic parameters we found a rapid normalization of liver lactate and LP ratio in patients with an uneventful course in accordance with a previous study (89). The higher LP ratio in subcutaneous tissue reflected low values of lactate and even lower values of

pyruvate, and was not a sign of ischemic metabolism since there was no increase in lactate values.

Stable, but somewhat higher levels of glucose was seen in the liver compared to subcutaneous tissue. In the experimental shock study, we observed higher baseline values for glucose in the liver, which increased with progressive blood loss. However, when blood loss exceeded the anaerobic threshold glucose rapidly decreased. Increased liver glucose during ischemia has been reported previously and can be explained by increased glycogen degradation (131).

In uncomplicated patients glycerol was found to be higher subcutaneously than in the liver. Increased adrenergic stimulation due to the surgical and postoperative stress, split triglycerides to fatty acids and glycerol, and may explain the higher subcutaneous values postoperatively.

After pilot studies we regarded IL-6, IL-8, MCP-1 and IP-10 and the complement split product C5a as relevant and recoverable inflammatory mediators. These were analyzed on day 1, 3, 5 and 7. Due to limited resources, C5a was only analyzed in two patients with complications and two patients without complications.

We found a rise in IL-6 immediately after transplantation, normalizing at day 3. This finding is in accordance with previous findings and may represent a minor local inflammatory reaction to insertion of the catheter. In uncomplicated patients low and stable IL-8 and MCP-1 values were detected.

In ”normal” patients we found, somewhat surprisingly, a steady increase of IP-10 median values in the liver (ranging from about 200 picog/ml to 3000 picog/ml with large variance), but not in subcutaneous tissue. It may reflect a liver normal regeneration process or a subclinical ”mini-rejection”, involving parts of the inflammatory network of which IP-10 is a marker. “Normal” patients were defined as patients without major clinical complications

during the time the catheters were inserted. This did not exclude that some of patients had subclinical rejection with somewhat increased liver enzymes or bilirubin not high enough to justify a liver biopsy. Our main hypothesis was therefore that increased IP-10 in normal patients most probably reflected subclinical mini-rejections. This is in accordance with the observation that with clinical biopsy verified rejection, IP-10 was 10 – 50 fold higher than in the normal population and that an increase in IP-10 probably reflects rejections at different degrees.

Recently, our group finished a larger study of 70 liver transplanted patients and a total of 73 transplantations (132;133). A longer registration time was conducted with microdialysis measurements for 9 (0-26) days. Thirteen patients had histologically verified rejections and nine patients developed hepatic artery occlusion. Forty patients were without major complications, and inflammatory parameters were measured for the 17 patients with least fluctuations in s-bilirubin and s-ALT. In line with our measurements in study III, IP-10 stayed at low values in “uncomplicated” patients, but increased up to 20 times in patients with a histologically verified rejection. Interestingly, patients with ischemia due to HAT showed low IP-10 levels as the uncomplicated patients. So IP-10 stands out as a promising and specific parameter of rejection.

After reperfusion, lactate rapidly normalized. A late increase in lactate may represent ischemia or increased metabolism, as described above. In ischemic tissue the LP ratio will rise because of the rise in lactate and the drop in pyruvate. In two ”normal” patients we saw a transient increase in lactate and the LP ratio which paralleled decreased flow and higher resistive index in hepatic artery.

Case 3 with HAT, developed a rapid rise in lactate and a rise in L/P ratio to 60, confirming an ongoing ischemia. In the larger clinical follow-up study (see above) a much higher rise in lactate was seen in patients with HAT than rejection, indicating that lactate and pyruvate

stands out as the most useful parameters of ischemia. Importantly it provides a continuous monitoring, as opposed to the cytokine measurements for which we have no ”fast-track” analyzer system.

Patients with rejection had a rise in intrahepatic lactate and pyruvate indicating a hypermetabolic state. A marked increase in inflammatory mediators was found, suggested a rejection, which later was histologically verified by a biopsy. The hypermetabolic state is most likely caused by T-cells and other cells from the immune system that with activation increases the metabolism several fold. Despite oxygen being present, the increased aerobic glycolysis induces a spill-over of lactate *and* pyruvate with increased values of both. Thus LP-ratio does not increase as opposed to events of ischemia.

Case 1 and 2 developed graft rejection. They demonstrated high values of C5a, but normal IL-8 immediately after the transplantation. C5a normalized transiently, but increased later in parallel to IL-8 several days before the rise of s-ALT and s-bilirubin suggesting liver damage. In studies by Silva et al (84;134) high levels of lactate and intrahepatic complement activation were both predictive of increased IRI and initial poor graft function. All the three cases suffering from rejection showed a 10 to 50 fold rise of IL-8. However, IL-8 does not seem to be specific for rejection. In the large follow-up study, referred to above (132;133) , IL-8 was more elevated after HAT than after rejection. In another study IL-8 was found to be higher after infection than after rejection (135). To make a diagnosis IL-8 needs to be compared to other parameters and is therefore less specific for rejection than IP-10.

For patients undergoing a liver transplantation microdialysis may provide early valuable information during the first postoperative week to diagnose ischemia or rejection. This can be of great practical help especially after HAT and acute rejection when early intervention is needed.

Conclusions:

Hemorrhagic shock model

- Organ blood flow reduction, caused by bleeding, varies widely between different organs with the kidney being the first to attain zero blood flow, while the intestine has the highest macrovascular flow
- $PtCO_2$ increases 1 – 4 kPa at a rate of 0,30 – 0,72 kPa/hour with decreasing blood supply even when the metabolism is aerobic in contrast to metabolic parameters of ischemia that does not change significantly
- $PtCO_2$ increases rapidly with the onset of anaerobic metabolism in all studied organs with anaerobic threshold values of median 7.7 – 9.3 kPa and a rate of increase of 3.7 – 9.7 kPa/hour i. e. more than tenfold the rate compared to aerobic conditions
- Under anaerobic conditions both $PtCO_2$, tissue lactate, LP ratio and tissue glycerol increase profoundly, while tissue pH and bicarbonate decrease strongly, indicating CO_2 generation caused by tissue metabolic acidosis buffered by bicarbonate
- $PtCO_2$ can be used as a marker of tissue hypoperfusion under both aerobic and anaerobic conditions. It gives an earlier warning of hypoperfusion than metabolic markers, and increases concomitantly with or earlier than other markers at the onset of tissue anaerobiosis

In vitro microdialysis study

- 20 kDa microdialysis catheters had zero recovery of a panel of cytokines, chemokines and complement factors, despite the fact that nearly all the tested substances had molecular weight less than 20 kDa
- 100 kDa catheters recovered all the immunological substances, but at very different degrees

- Maximum recovery was for most substances at a rate of 1 μL/min and we therefore decided to use this rate in the clinical study

Clinical microdialysis study on liver transplanted patients

- Metabolic parameters (glucose, lactate, pyruvate, glycerol) and immunological parameters (IL-6, IL-8, MCP-1, IP-10) and complement factor C5a was recovered in 100 kDa microdialysis catheters during 7 days postoperatively.
- In patients without complications lactate in the liver decreased rapidly and attained normal values within 24 hours. For the next six days values were relatively stable.
- Lactate in the liver was higher than lactate subcutaneously
- LP ratio and glycerol was higher in the subcutaneous tissue than in the liver
- IL-6 was higher subcutaneously than in the liver the first postoperative days, but was not different from liver values from days 3
- IP-10 increased in the liver, but not subcutaneously during the observation period
- In a patient with rejection there was a substantial increase in lactate, pyruvate, IP-10, IL-8 and C5a four days before increase in ALT and four days before rejection was biopsy verified. Anti-rejection treatment with high dose methylprednisolone was started and all measured parameters rapidly declined
- In a patient with hepatic artery thrombosis, a rapid rise in lactate and glycerol was demonstrated

Further perspectives

We have outlined the theoretical mechanisms and showed in an experimental animal study that PCO_2 has a great potential in monitoring hypoperfusion both under aerobic and anaerobic

conditions. A PCO_2 sensor with real-time read-out has been developed by our group for tissue measurements and approved for human use. In experimental animal studies the sensor was able to detect ischemia in heart, liver, intestine, kidney and skeletal muscle within 1 – 5 minutes. This may become a valuable tool for the clinician in early detection and treatment of organ ischemia.

Our research group has carried out a study on 70 liver transplant patients after the completion of study III in this thesis. In 73 transplanted livers, there were 20 rejection when catheters were implanted (up to 28 days, median 9 days), and 9 patients with ischemia. Ischemia was detected by 100% sensitivity and specificity, and rejection with > 84% sensitivity and > 90% specificity. These results were achieved by measuring lactate and pyruvate bedside by sampling every 1 – 2 hour. Ischemia was detected close to real time, and rejection was detected 2 – 5 days before increase in ALT or bilirubin. No complications related to the catheters were detected. We have now extended this study in children by also placing a 100 kDa catheter in the abdominal cavity between intestinal loops. In this way we have detected portal vein thrombosis (venous ischemia in intestine), intestinal perforation and peritonitis. We have also in pilot patients inserted microdialysis catheters percutaneously into the peritoneal cavity between intestinal loops under ultrasound guidance. This has been in patients at risk of abdominal compartment syndrome and in septic patients on high doses of pressors. In one patient with high doses of norepinephrine we detected intestinal ischemia. By decreasing the dose, accepting a lower MAP and adding dobutamine, the intestinal ischemia was reversed. We are currently planning studies to use abdominal microdialysis to guide fluid and vasoactive treatment in patients with shock (hypovolemic, cardiac, septic) to prevent intestinal ischemia. Also a study where we aim to detect postoperative infections after abdominal surgery earlier than with current monitoring is planned. In this study we will place

catheters near intestinal anastomosis, and in the surgical wound. We have in animal studies (cecal ligation model) found that this approach detect infection very early.

The current thesis has been hypothesis generating for later performed and planned studies. It is our hope that this will benefit our patients implying reduced suffering and better outcome.

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I

Tissue gas tensions and tissue metabolites for detection of organ hypoperfusion and ischemia

L. WÆLGAARD[1], B. M. DAHL[2], G. KVARSTEIN[1], T. I. TØNNESSEN[1,2,3]

The Acute Clinic; Department of Anesthesiology and Critical Care Medicine[1] and the Intervention Centre[2], Oslo University Hospital - Rikshospitalet, and Faculty of Medicine[3], University of Oslo, Norway

Corresponding author:

Tor Inge Tønnessen

Oslo University Hospital - Rikshospitalet

Box 4950 Nydalen

N-0424 Oslo, Norway

Tel: +4723070000

Fax: +4723073681

E-mail: t.i.tonnessen@medisin.uio.no

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Background: The aim of this study was to evaluate how tissue gas tensions and tissue metabolites measured *in situ* can detect hypoperfusion and differentiate between aerobic and anaerobic conditions during hemorrhagic shock. We hypothesized that tissue PCO_2 ($PtCO_2$) would detect hypoperfusion also under aerobic conditions, and detect anaerobic metabolism concomitantly with, or earlier than other markers.

Methods: Prospective experimental animal study with eight anesthetized pigs subjected to a continuous blood loss ~8 % of total blood volume per hour until death. We measured cardiac index (CI), organ blood flows, and tissue levels of PO_2, PCO_2, glucose, pyruvate, lactate and glycerol in intestine, liver, kidney and skeletal muscle.

Results: With reduction in blood flow to the organs under aerobic conditions, $PtCO_2$ increased ~1 - 4 kPa from baseline. With the onset of tissue hypoxia there was a pronounced increase of $PtCO_2$, lactate, lactate-pyruvate (LP) ratio and glycerol. Tissue pH and bicarbonate decreased significantly, indicating that metabolic acid was buffered by bicarbonate to generate CO_2.

Conclusion: Moderate tissue hypoperfusion under aerobic conditions is associated with increased $PtCO_2$, in contrast to metabolic parameters of ischemia (lactate, LP ratio, glycerol) which remain low. From the onset of ischemia there is a much more rapid and pronounced increase in $PtCO_2$, lactate and LP ratio. $PtCO_2$ can be used as a marker of hypoperfusion under both aerobic and anaerobic conditions; it gives an earlier warning of hypoperfusion than metabolic markers, and increases concomitantly with or earlier than other markers at the onset of tissue anaerobiosis.

Severe bleeding due to major trauma or surgery is a serious condition with mortality rates as high as 30 %.[1] Optimal resuscitation is difficult to achieve since systemic parameters like blood pressure and heart rate do not reliably reflect blood flow to internal organs.[2-5] Visceral organs like the liver, intestine and the kidney may for instance be hypoperfused while systemic variables are still close to normal.[6-9] Several authors have therefore advocated measuring ischemia markers in the organs to detect if O_2 supply is sufficient to support the O_2 demand.[10-12]

Tissue PCO_2 ($PtCO_2$) as a marker of hypoperfusion is supported by previous studies.[13-20] Gastric tonometry has been shown to detect gastric hypoperfusion during shock.[3;4;11;21] Microdialysis is a clinically available technology that can be used in muscle and abdominal organs like the liver and intestine to detect ischemia. [22-26] There are also emerging clinical technologies for real-time measurement of PCO_2 in muscle and internal organs.[27-29] The majority of PCO_2 studies have been carried out in different parts of the GI tract, mainly stomach, and less is known about PCO_2 in solid organs during hemorrhagic shock.[18;20;30;31] No studies have compared real-time $PtCO_2$ in several organs with other simultaneously measured microdialysis tissue markers of ischemia like lactate, lactate-pyruvate ratio (LP ratio) and glycerol. In this exploratory study we used a model of continuous hemorrhage with measurements of organ blood flow as well as tissue PCO_2, PO_2, pH, lactate, pyruvate, glucose and glycerol in skeletal muscle, kidney, liver and intestine.

The aim of this study was to evaluate how tissue gas tensions and tissue metabolites measured *in situ* as well as systemic parameters can detect hypoperfusion and differentiate between aerobic and anaerobic conditions during hemorrhagic shock. We hypothesized that $PtCO_2$ could detect hypoperfusion also under aerobic conditions, and detect anaerobic metabolism concomitantly with, or earlier than other markers depending on the organ studied.

Materials and methods

The study protocol was approved by the Institutional Committee of Animal Care and Use and performed according to international guidelines [32]. Eight male Norroc pigs with a median weight of 60 kg (range 53-71 kg) were included.

The animals were kept fasting overnight with free access to water and were sedated with i.m. ketamine (20 mg/kg), azoperon (3mg/kg) and atropin (0.02 mg/kg). Anesthesia was induced by i.v. penthobarbital (4 - 5mg/kg) and morphine (0.2 mg/kg) and maintained with isoflurane inhalation 1.0-1.5 % and infusion of morphine (0.15-0.2 mg/kg/h).

Surgery

A tracheostomy was performed and mechanical ventilation established at 7 – 10 L/min at a respiratory rate of 14 per min and tidal volumes of 0.5 – 0.7 L aiming at normocapnia (~5.3 kPa). (Kion™, Siemens AB, Solna, Sweden). Inspired oxygen fractions (FiO_2) were between 0.25 – 0.35 to attain normoxia (pO_2 >12 kPa). PEEP was zero. A three luminal catheter was introduced in the carotid artery for continuous arterial blood pressure recording, blood sampling and controlled bleeding. A multilumen, fiberoptical pulmonary artery catheter (CCombo™, Edwards Lifesciences LLC, Irvine,CA) was inserted to record cardiac index, SvO_2, and core temperature. Cardiac index was determined by continuous thermodilution which has a delay of 9 – 11 minutes until the results appear. In our graphs we have compensated for this time delay.

Through a midline laparotomy dissection of the portal vein, hepatic artery, descending aorta and inferior caval vein was performed and catheters were introduced in the portal vein, hepatic vein and inferior caval vein for extraction of blood samples. Transit time ultrasound flow-

probes (Medistim AS, Oslo, Norway) were placed around the portal vein, the hepatic artery and the descending aorta, immediately proximal to the bifurcation of the iliac arteries.

Through a flank incision from iliac crest to 12th rib and after dissection of the kidney we introduced a short catheter (Venflon™, Viggo 1.0 mm Ø) into the left renal vein and a transit time ultrasound flow probe around the renal artery.

The following measurement devices were used: Neurotrend™ Multiparameter Sensor tissue monitoring system (Diametrics, Inc. 2658 Patton Rd. Roseville, MN 55113, USA) was inserted into the tissue of the liver and the left kidney to measure $PtCO_2$, tissue PO_2 (PtO_2) and tissue pH. Via an antimesenteric incision of the small intestine two Severinghaus type PCO_2-electrodes (MI 720™, Microelectrodes, Bedford, NH) were placed in the lumen. Skeletal muscle PCO_2 was measured in the gluteal muscle. Intestinal lumen and skeletal muscle PO_2 was monitored by an oxygen electrode (Licox™, Integra, Plainsboro, NJ, USA). Microdialysis catheters (CMA 60 MD Catheter, CMA Microdialysis AB, Solna, Sweden) were inserted into the gluteal muscle, kidney, liver and the intestinal lumen.

To compensate for current fluid loss Ringer's Acetate 10ml/kg/h was administered during surgery. Core temperature was kept at 38ºC±1 using a temperature regulating blanket. An infusion of glucose 10 % was given to maintain blood glucose 4 – 8 mM throughout the experiment.

Protocol for hemorrhagic shock

In pilot experiments we tested animals (n > 10) that underwent major abdominal surgery with equivalent anesthesia and with insertion of intraorgan measurements of the same parameters as used in this study, but no bleeding protocol was started. These animals were observed for 12

hours after surgery, and showed no change in cardiovascular parameters, or in any of the parameters used for systemic or intraorgan detection of ischemia. The drift of the measurement devices were minimal. Based on this, we decided both for ethical and economic reasons that a control group was not deemed necessary in this study, and each animal could serve as its own control.

After the end of surgery the animals were allowed to stabilize for one hour. During this hour three samples of baseline values of all parameters confirmed no changes in baseline values showing that the pigs were stable after surgery. The protocol started with another two sets of baseline values during ten minutes. With a volume pump (IVAC™ 598, Cardinal Health Inc., Ohio, USA) we performed a continuous withdrawal of blood from the carotid artery catheter corresponding to ~8% of the total blood volume every hour. Blood volume was calculated as 65 mL/kg body weight.[33] There was no infusion of crystalloids or colloids while the withdrawal of blood was ongoing until death.

Data Collection

$PtCO_2$, PtO_2, tissue pH, tissue HCO_3^- and temperature from the Neurotrend, $PtCO_2$ from the Severinghaus-type sensors and PtO_2 from the Licox™ probes were recorded every 60 seconds. Every 30 minutes, microdialysates were collected and analyzed by a CMA 600 Microdialysis Analyzer for tissue levels of lactate, pyruvate, glycerol and glucose. Blood samples were simultaneously drawn from the carotid artery, renal vein, hepatic vein, portal vein and inferior caval vein and analyzed immediately (Radiometer ABL 625, Copenhagen, Denmark).

Calculations and statistics

To determine transition to anaerobic metabolism we aimed at defining the cardiac index value (critical cardiac index, CI_{crit}) when the rapid rise in lactate, LP ratio, $PtCO_2$ and glycerol commences, and tissue pH, bicarbonate and glucose declines. This corresponds to the maximal curvature of the graph, which can be determined by dual line regression analyses.[34] Thus, we carried out dual line regression analyses with cardiac index on the x-axis and each variable on every animal in the organs studied on the y-axis. Critical CI was defined as the point of intersection for the two linear regression lines with the lowest sum of squared residuals.[34] This rapid increase or decrease in parameters reflecting anaerobic metabolism occurs near the transition to anaerobic metabolism, and we therefore propose that CI_{crit} can be used as parameter for commencement of tissue hypoxia. Each variable is presented as median with interquartile range (25% - 75%). To test for significant differences between the critical CI values, we applied Kruskal-Wallis One Way Analysis of Variance on Ranks, and the post-hoc Student-Neuman Keuls (SNK) test for a multiple comparison of all the groups.

We also calculated the time derivates, $dPCO_2/dT$(time) and d[lactate]/dT, under aerobic and anaerobic conditions for the investigated organs. This gives information on the rate of increase of these parameters. The rapid increase in time derivate commenced at the critical CI value, adding to the argument that CI_{crit} reflects the anaerobic threshold.

Areas under the flow curves (AUC) (Fig. 2) were calculated using the sum of rectangles method. To test if there were significant differences between the AUC of blood flow in different organs, we applied Kruskal-Wallis One Way Analysis of Variance on Ranks, and the post-hoc Student-Neuman Keuls (SNK) test for a multiple comparison of all the groups.

In one animal the renal venous catheter and in another the renal microdialysis catheter was obliterated. In two animals the intestinal microdialysis catheter failed. LP ratio was not calculated for the intestine due to some values of pyruvate being lower than the detection limit.

Results

Cardiac index (CI) decreased with increasing blood loss and showed a strong inverse linear correlation ($r > -0.95$ in each pig). Heart rate increased at CI of 4000 mL/kg/m^2 reaching maximal rate at ~3000 mL/kg/m^2 (Fig. 1A). From baseline to ~3000 mL/kg/m^2 MAP decreased ~5 mmHg per liter decrease in CI. Thereafter MAP had a steeper decline at ~15 mmHg/L decay in CI (Fig. 1B). CI is in Fig. 2 compared with organ blood flows expressed as percentage decrease from baseline. Blood flow to the kidney ($p < 0.01$) and skeletal muscle ($p<0.01$) was relatively more reduced than cardiac output, while intestinal (portal) blood flow was better preserved ($p< 0.05$). Hepatic artery blood flow decreased to the same degree as CI.

PtO_2 declined in all studied organs in the CI range from baseline to ~3000mL/min/m^2 (Fig. 3) at a rate of ~0.4 – 0.5 kPa/L decrease in CI. With further blood loss PtO_2 decline was more pronounced (~1.3 – 1.8 kPa/L) until it reached zero. Zero PO_2 was attained significantly earlier in the kidney than in the other organs ($p < 0.02$).

Under aerobic conditions tissue LP ratio, glycerol and lactate remained low in all organs (Fig. 3 and 4, Table 2), but increased significantly and at a much higher rate below critical CI levels (CI_{crit}) (Table 1 and 2, Fig. 3 and 4).

Only $PtCO_2$ increased significantly under both aerobic and anaerobic conditions (Fig 4, Table 1 and 2). In all organs and for all variables the rate of increase was significantly and several fold higher under anaerobic compared to aerobic conditions (Fig. 3, Fig 4 and Table 2).

In all organs CI_{crit} for LP ratio were significantly lower than for PCO_2 and lactate (Table 1). Glycerol increased at a significantly lower CI_{crit} ($p < .01$) than LP ratio in all organs tested. CI_{crit} for glycerol and LP ratio did not significantly differ between the organs. However, for lactate it was significantly lower ($p < .05$) in muscle compared to the other organs. There was no significant difference in critical CI values between PCO_2, lactate, pH and bicarbonate in kidney and liver. In muscle and intestine CI_{crit} for lactate was significantly lower than for PCO_2. Arterial lactate reached pathological values (> 2 mM) at CI ~2050 mL/kg/m^2, whereas arterial pH, bicarbonate and BE reached values significantly lower than baseline at CI ~1900 mL/kg/m^2.

Discussion

We found that i) organ blood flow reduction, caused by bleeding, varies widely between different organs with the kidney being the first to attain zero blood flow, while the intestine has the highest macrovascular flow; ii) $PtCO_2$ increases 1 – 4 kPa at a rate of 0,30 – 0,72 kPa/hour (Table 1 and 2) with decreasing blood supply even when the metabolism is aerobic in contrast to metabolic parameters of ischemia that does not change significantly; iii) $PtCO_2$ increases rapidly with the onset of anaerobic metabolism in all studied organs with anaerobic threshold values of median 7.7 – 9.3 kPa and a rate of increase of 3.7 – 9.7 kPa/hour i. e. more than tenfold the rate compared to aerobic conditions; iii) Under anaerobic conditions both $PtCO_2$, tissue lactate, LP ratio and tissue glycerol increase profoundly, while tissue pH and bicarbonate decrease strongly, indicating CO_2 generation caused by tissue metabolic acidosis buffered by bicarbonate.

Phases during hemorrhagic shock

With decreasing cardiac index due to increasing blood loss we found that organ metabolism progressed through two phases:

Phase 1 starts at baseline and ends at the critical CI level (CI_{crit}). The metabolism is solely aerobic, and the phase is characterized by a progressive decrease in PtO_2 [9;31;35-37] and an increase in $PtCO_2$, but no significant changes in tissue lactate, LP ratio, tissue glycerol or glucose in any organ or systemic blood (Fig. 3 - 4, Table 1 – 2).

As blood flow declines, more oxygen is extracted from hemoglobin to keep a constant oxygen utilization (VO_2) and CO_2 generation (VCO_2) in the tissue. With decreased blood flow more CO_2 is consequently added to each volume unit of blood, and PCO_2 will increase in venous effluent blood as well as in the tissue [36;38;39]. Thus, the increase in $PtCO_2$ under aerobic conditions is reflecting decreased blood flow (hypoperfusion/stagnation), and not increased aerobic VCO_2.

Phase 2 starts at the transition from solely aerobic metabolism to mixed aerobic and anaerobic metabolism and continues until the subject dies. As CI decreases below the anaerobic threshold a higher percentage of the metabolism becomes *an*aerobic, and when PtO_2 is zero (anoxia) the metabolism has become purely anaerobic. The phase is characterized by marked *increases* in the tissue levels of PCO_2, lactate, LP ratio and glycerol, and considerable *decreases* in tissue pH, bicarbonate and glucose.

Under anaerobic conditions protons are released from lactic acid, ATP hydrolysis, and dissociated phospholipids [15]. Protons are buffered by HCO_3^- to generate CO_2 [37;40]. In accordance with this, not only pH, but also bicarbonate decreased markedly demonstrating a tissue metabolic acidosis (Fig. 4, Table 1). We found a high correlation between lactate and $PtCO_2$ in ischemic tissue (below CI_{crit}) in accordance with previous findings under no-flow conditions [15;18;41]

compatible with lactic acid being buffered by bicarbonate. The amount of CO_2 generated (VCO_2) under anoxic conditions is less than under aerobic conditions [3;40;42-45], but since CO_2 is trapped in the tissue with minor blood supply, tissue PCO_2 increase several fold despite lower VCO_2.

Determination of transition to anaerobic metabolism

Lactate-pyruvate ratio is a robust marker of anaerobic metabolism as this ratio reflects the redox state of the cell ($NADH \times H^+/NAD^+ = (Lactate/Pyruvate) \times K_{LDH}$)) and shows that pyruvate is being utilized to produce lactate; a sign of anaerobic glycolysis.[46] Interestingly, increasing LP ratio was found at significantly lower CI_{crit} in all studied organs than lactate and $PtCO_2$ (Table 1). This was because pyruvate initially increased when lactate increased, but pyruvate then decreased a lower CI. Since LP ratio, lactate and $PtCO_2$ increase at different CI_{crit}, which of these parameters reflects transition to anaerobic metabolism? In liver and kidney the CI_{crit} for tissue PCO_2, lactate, pH and bicarbonate are nearly equivalent which strongly indicates that these markers reflect the imminent ischemia (Table 1), LP ratio increases at CI_{crit} only 300 $mL/kg/m^2$ lower. Rather than speculating on which variable that most truly reflects an anaerobic threshold, we emphasize that despite CI_{crit} being significantly different for various markers the difference in values are rather small (Table 1) indicating that they reflect the same biological phenomenon. From a clinical point of view it would be logical to increase resuscitative efforts to prevent ischemia when the first marker ($PtCO_2$) starts to increase and not waiting until LP ratio also increases.

Only glycerol, which is a breakdown product of cell membrane lipids indicating severe cell injury or cell death, increases at a lower CI_{crit} than LP ratio. This is in accordance with the

notion that ischemia may last for some time before cell damage occurs, leaving time for initiation of therapy based on early ischemia markers like $PtCO_2$ at a time when the cells are salvageable.

Organ blood flow and tissue ischemia

Splanchnic blood flow has been considered particularly vulnerable to hypovolemia. In our study, however, blood flow to the liver and intestine was surprisingly well preserved with increasing blood loss. When blood flow to the kidney was zero, blood flow to the intestine was not reduced by more than 50% (Fig. 2). However, in the kidney transition to anaerobic metabolism did not take place until blood flow almost reached zero, whereas signs of intestinal anaerobic metabolism were found at 50% of baseline blood flow (Fig. 2 – 4). These findings illustrate clearly that macrovascular flow at organ level does not reliably reflect microvascular blood flow and the transition to anaerobic metabolism[5], and is as such of very limited value to differentiate serious blood loss from non-threatening blood loss. In that perspective intraorgan monitoring of tissue metabolites and gas tensions is preferred.

Are systemic parameters sensitive enough to detect organ ischemia?

The first signs of tissue ischemia occurred at SvO_2 ~40, indicating that for hypovolemic shock a resuscitation target of SvO_2 50 % would suffice. Arterial pH, bicarbonate, BE and lactate reached values significantly different from baseline at CI ~1900 – 2000 mL/kg/m^2 which is significantly lower than the CI_{crit} we detected by tissue monitoring (Table 1 and Fig. 3 - 4). Thus, anaerobic metabolism in abdominal organs may be present despite normal arterial levels of these variables.

The animals became tachycardic in the aerobic phase, and reached maximum heart rate when the organs became ischemic (Fig. 1). With a MAP > 50 mmHg there were no signs of

tissue ischemia, supporting clinical practice in resuscitation targeting MAP ~60 mmHg. However, if the patient has arteriosclerotic disease in the aorta or vessels supplying abdominal organs, or maldistributive shock, organ ischemia may occur at MAP 50 - 60 mmHg supporting the need for intraorgan monitoring.

Putative clinical implications of this study

Microdialysis catheters are clinically approved for monitoring liver[22;26], intestine and skeletal muscle, and a PCO_2 sensor [24-26] is FDA approved and CE marked for clinical use. We have carried out studies on liver transplant patients where we have inserted microdialysis catheters in transplanted livers and detected ischemia due to vessel thrombosis close to real time enabling reoperation at a time before irreversible injury occurred.[26] Intestinal ischemia is detected by these devices when they are placed between intestinal loops, eliminating need to penetrate the intestinal wall. [25] Placement of the intraabdominal sensors can then be carried out through a paracentesis needle in less than 5 min. Thus, it is feasible to perform clinical studies with these devices in patients at risk of hemorrhagic shock both for measurements in skeletal muscle and abdominal cavity. PCO_2 in skeletal muscle closely correlated with PCO_2 in abdominal organs. Muscular PCO_2 could therefore serve as a surrogate marker for these organs. In addition to early detection of ischemia, these devices can be used to guide therapy, as resuscitation to a cardiac index above CI_{crit} would rapidly normalize intraorgan parameters. This could be of particular benefit for patients with stenotic abdominal vessels, and for patients being treated with vasopressors like noradrenalin[47] and vasopressin[48] that may decrease microcirculation in abdominal organs despite elevation of MAP in shock.

Limitations

The study is carried out on healthy anesthetized animals. Therefore the data cannot be extrapolated to awake individuals or patients with severe atherosclerosis, but most patients being treated for severe blood loss are heavily sedated or anesthetized, so our study design may be realistic from a clinical perspective.

For measurements of gas tensions, we have used Neurotrend in kidney and liver, and Severinghaus type sensors for PCO_2 in muscle and intestine, and a Clark-type sensor for PO_2 in the latter organs. Thus, comparing tensions in intestine and muscle with liver and kidney could induce erroneous conclusions if the accuracy of the different methods were not comparable. To avoid this, we meticulously calibrated all devices before and after the experiment against known values of PCO_2 and PO_2 and developed algorithms for drift correction if needed. We therefore feel confident that despite using different devices our findings and conclusions are valid.

Conclusion

$PtCO_2$ can be used as a marker of tissue hypoperfusion under both aerobic and anaerobic conditions. It gives an earlier warning of hypoperfusion than metabolic markers, and increases concomitantly with or earlier than other markers at the onset of tissue anaerobiosis.

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Conflicts of interest: None

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Legend to figures

Figure 1

Cardiac index vs. heart rate, mean arterial pressure and mixed venous oxygen saturation during progressive blood loss. Values are medians with interquartile ranges.

Figure 2

Cardiac index (CI) vs. blood flow during progressive blood loss. Blood flows are shown as percentage change from baseline values. The grey dashed line represents median percentage reduction in cardiac index. Values are medians with interquartile ranges.

Figure 3

Tissue PO_2 and tissue values of glycerol, glucose and the calculated lactate-to-pyruvate ratio (LP-ratio) vs. cardiac index (CI). Values are medians with interquartile ranges. When error bars are not visible, the interquartile ranges are less than the size of the symbol.

Figure 4

Tissue values of PCO_2, lactate, pH and bicarbonate vs. cardiac index (CI). Values are medians with interquartile ranges. When error bars are not visible, the interquartile ranges are less than the size of the symbol. Lactate-pyruvate ratio for intestine is not depicted due to some values of pyruvate being less than detectable by CMA 600.

Figure 1

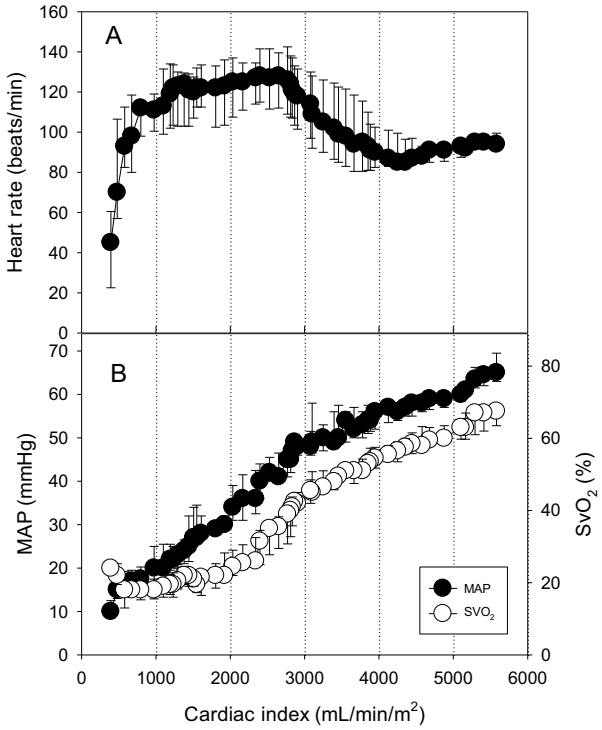
A
B
Heart rate (beats/min)
MAP (mmHg)
SvO2 (%)
Cardiac index (mL/min/m2)
MAP
SVO2
0
20
40
60
80
100
120
140
160
10
30
50
70
1000
2000
3000
4000
5000
6000

Figure 2

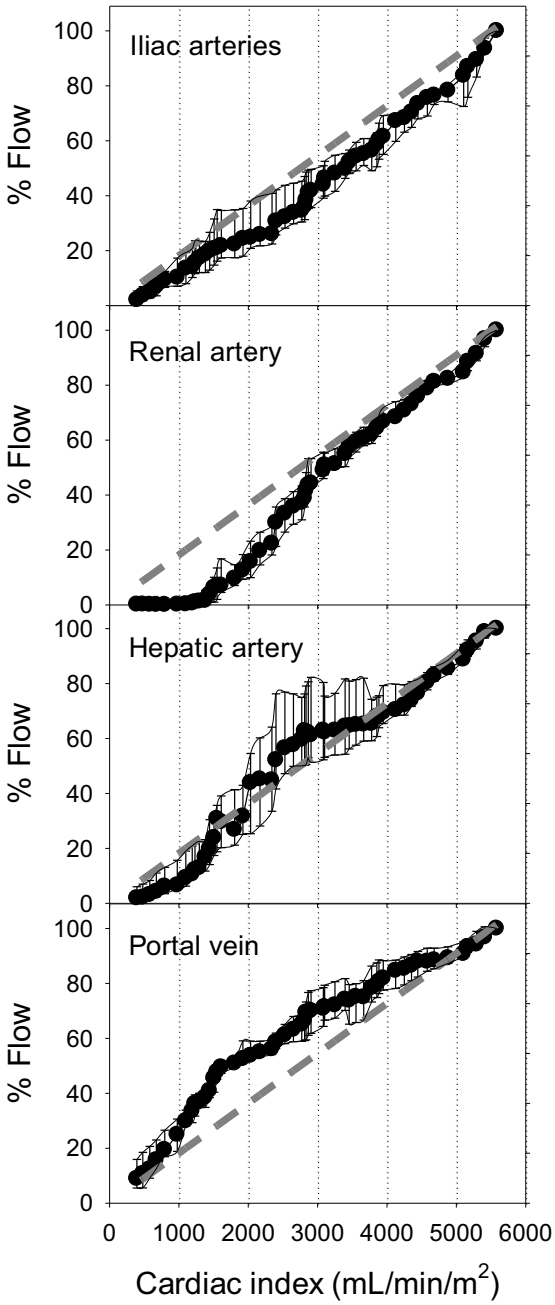
Iliac arteries
Renal artery
Hepatic artery
Portal vein
% Flow
0
20
40
60
80
100
0
1000
2000
3000
4000
5000
6000
Cardiac index (mL/min/m2)

Figure 3

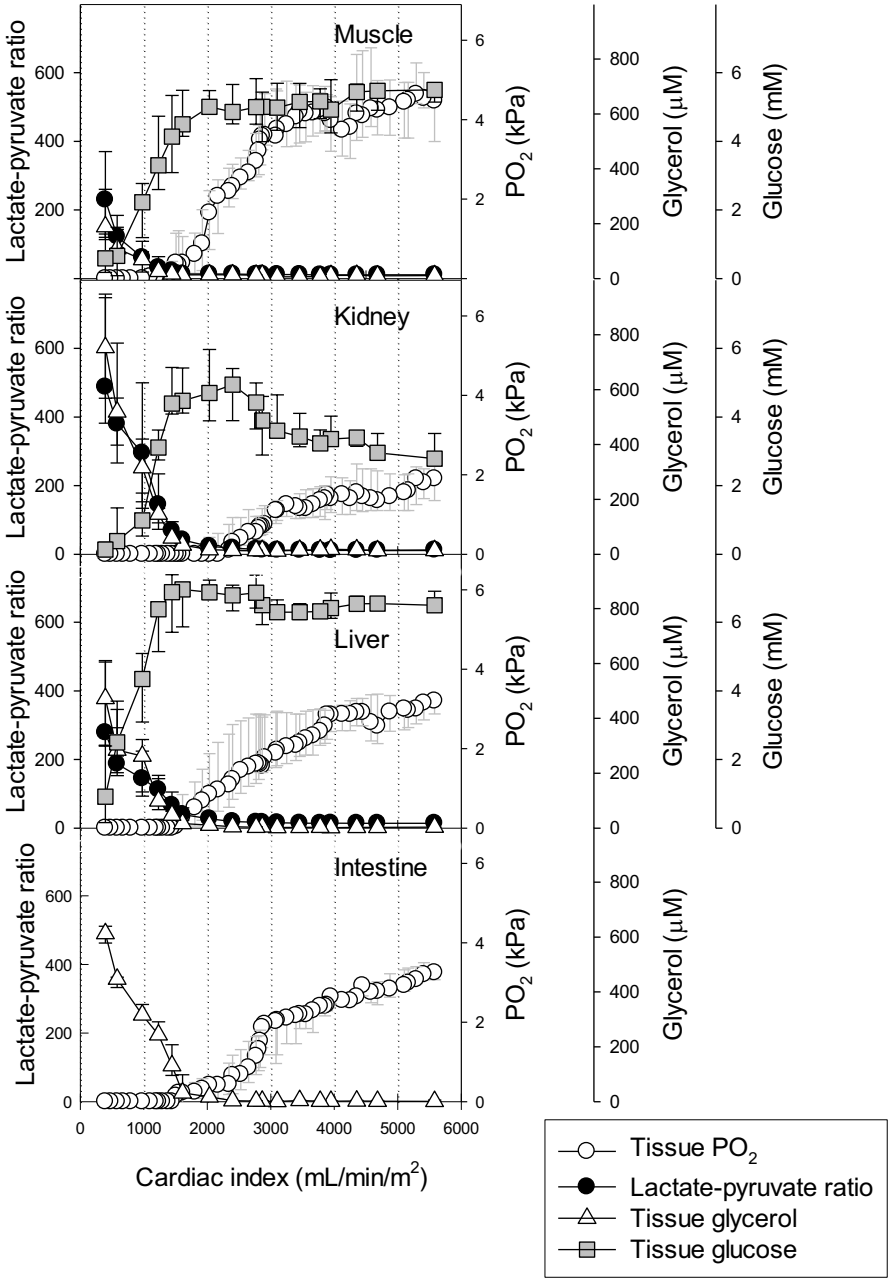
Muscle
Kidney
Liver
Intestine
Lactate-pyruvate ratio
PO2 (kPa)
Glycerol (μM)
Glucose (mM)
Cardiac index (mL/min/m2)
Tissue PO2
Lactate-pyruvate ratio
Tissue glycerol
Tissue glucose

Figure 4

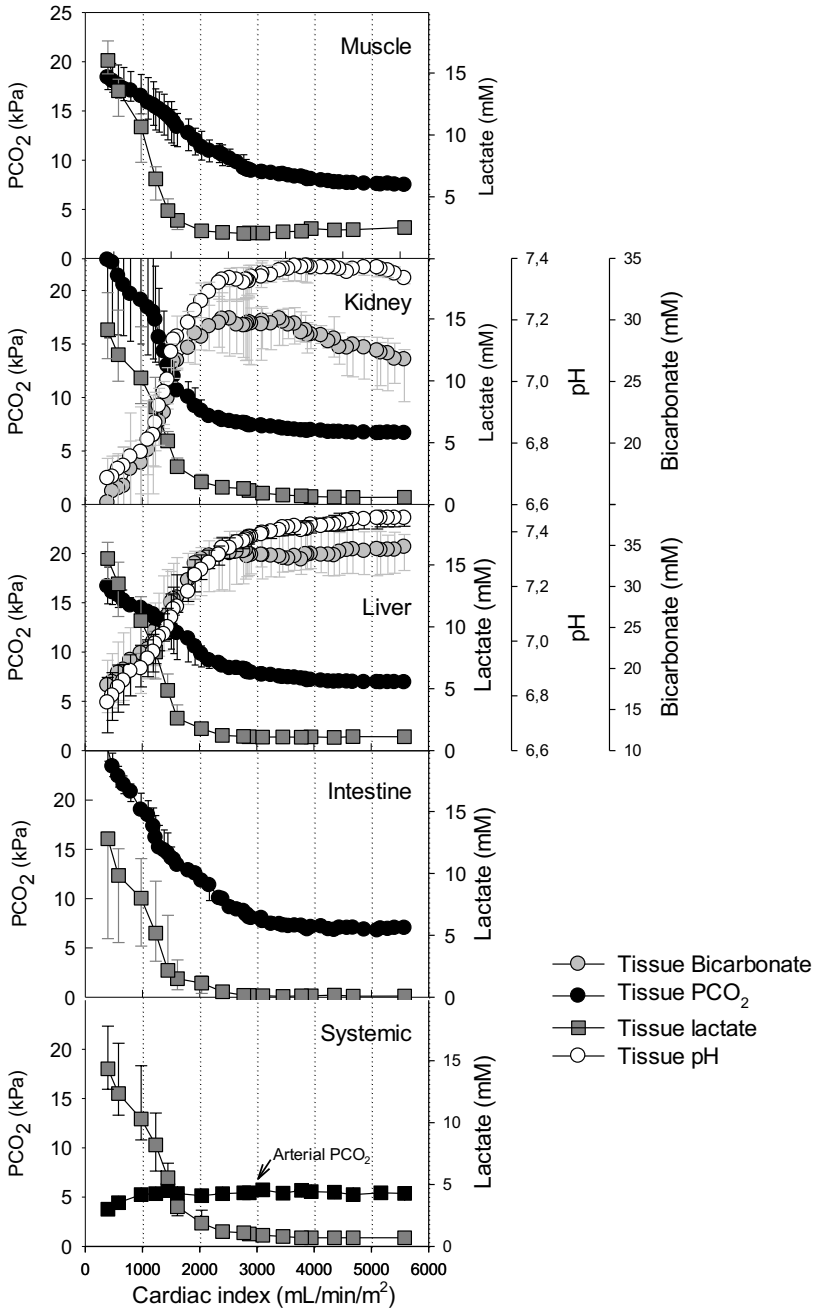
Muscle
Kidney
Liver
Intestine
Systemic
PCO2 (kPa)
Lactate (mM)
pH
Bicarbonate (mM)
Arterial PCO2
Cardiac index (mL/min/m2)
Tissue Bicarbonate
Tissue PCO2
Tissue lactate
Tissue pH

Table 1: Critical levels of cardiac index (CI_{crit}) and threshold values of variables

Parameter	Muscle		Kidney		Liver		Intestine	
	Cardiac index ($mL/min/m^2$)	Threshold value	Cardiac index ($mL/min/m^2$)	Threshold value	Cardiac index ($mL/min/m^2$)	Threshold value)	Cardiac index ($mL/min/m^2$)	Threshold value
Tissue PCO_2 (kPa)	2640 ★ (2326, 2814)	9.3 (8.9, 9.8)	2341 (2240, 2420)	7.7 (7.5,8.0)	2442 (2348, 2626)	8.5 (7.6,8.8)	2636 ★ (2440, 2740)	8.9 (8.2, 9.6)
Lactate (mM)	2002 # (1902, 2042)	2.1 (1.9, 2.2)	2290 (2050, 2530)	1.4 (1.2, 1.5)	2303 (2175, 2437)	1.2 (1.1,1.4)	2307 (1980, 2622)	0.2 (0.2,0,2)
LP ratio	1572 § (1496, 1749)	13.5 (12.9, 16.2)	1988 § (1883, 2003)	22.8 (19.2, 24.9)	1991 § (1902, 2052)	20.6 (19.3, 24.0)	NA	NA
Glycerol (µM)	1384 §§ (1268, 1421)	16 (8, 23)	1572 §§ (1494, 1734)	17 (15, 31)	1648 §§ (1566, 1667)	16 (9, 18)	1572 §§ (1521, 1610	20 (15,26)
pH	NA	NA	2376 (2116, 2635)	7.33 (7.30, 7.38)	2509 (2283, 2648)	7.38 (7.33, 7.41)	NA	NA
Bicarbonate (mM)	NA	NA	2177 (2199, 2395)	31 (29, 32)	2243 (2202, 2320)	33 (31, 35)	NA	NA

To determine the critical cardiac index value (CI_{crit}) for each variable, we carried out dual line regression analyses for each variable on every animal and in each organ studied. The threshold values of the variables are those found at the corresponding CI_{crit}. Tissue PCO_2 in muscle and intestine was significantly higher than in kidney and liver (★, $p < 0.01$). Muscle lactate was significantly lower than in other organs (#, $p < 0.02$). LP ratio (§, $p < 0.01$) was significantly lower in all organs compared to lactate and PCO_2. Glycerol (§§, $p < 0.01$) was significantly lower than LP ratio in all organs tested. To test for significant differences between the critical CI values, we applied Kruskal-Wallis One Way Analysis of Variance on Ranks, and the post-hoc Student-Neuman Keuls (SNK) test for a multiple comparison of all the groups.

Table 2: Rate of increase in PCO_2 and lactate under aerobic and anaerobic conditions

Parameter	Muscle		Kidney		Liver		Intestine	
	Aerobic	**Anaerobic**	**Aerobic**	**Anaerobic**	**Aerobic**	**Anaerobic**	**Aerobic**	**Anaerobic**
***d*PCO_2/*d*T (kPa hour^{-1})**	0.61 ★ (0.45, 0.80)	3.70 § (3.07, 4.12)	0.30 ★ (0.27, 0.37)	8.47 § (6.08, 13.02)	0.28 ★ (0.10, 0.42)	4.35 § (3.47, 6.15)	0.72 ★ (0.56, 0.82)	9.25 § (7.38, 10.70)
***d*Lactate/*d*T (mM hour^{-1})**	0.00 (0.00, 0,01)	6.65 § (6.47, 8.98)	0.11 (0.06, 0.17)	6.05 § (4.92, 7.15)	0.01 (0.00, 0.07)	6.80 § (5.47, 7.23)	0.03 (0.02, 0.17)	4.11 § (2.53, 5.57)

All values for aerobic *d*PCO_2/dT were significantly higher than zero (★, $p < 0.05$) whereas none of the aerobic *d*Lactate/*d*T was. Aerobic *d*Lactate/*d*T in the kidney, however, increased, but it did not reach significance ($p = 0.09$). All values for both parameters were significantly higher in the anaerobic phase compared to the aerobic (§, $p < 0.01$). Data are presented as median and interquartile ranges (25%, 75%). To test for significant differences between the groups, we applied Kruskal-Wallis One Way Analysis of Variance on Ranks, and the post-hoc Student-Neuman Keuls (SNK) test for a multiple comparison of all the groups.

II

III